

Open Research Online

The Open University's repository of research publications and other research outputs

Role of nitric oxide in the regulation of tumor necrosis factor-alpha signalling

Thesis

How to cite:

Barsacchi, Rico (2003). Role of nitric oxide in the regulation of tumor necrosis factor-alpha signalling. PhD thesis The Open University.

For guidance on citations see [FAQs](#).

© 2003 The Author



<https://creativecommons.org/licenses/by-nc-nd/4.0/>

Version: Version of Record

Link(s) to article on publisher's website:
<http://dx.doi.org/doi:10.21954/ou.ro.0000e813>

Copyright and Moral Rights for the articles on this site are retained by the individual authors and/or other copyright owners. For more information on Open Research Online's data [policy](#) on reuse of materials please consult the policies page.

oro.open.ac.uk

Rico Barsacchi

**Role of nitric oxide in the regulation of tumor
necrosis factor-alpha signalling**

Thesis submitted in partial fulfilment of the requirements of the Open
University for the degree of

Doctor of Philosophy
In
Molecular and Cellular Biology

January 2003

DIBIT
Department of Biological and Technological Research
San Raffaele Scientific Institute
Milan, Italy

**APPENDIX FROM PG 139 NOT
DIGITISED BY
REQUEST OF THE UNIVERSITY**

CONTENTS

CONTENTS	I
ILLUSTRATIONS AND TABLES	V
ABSTRACT	IX

Chapter 1: INTRODUCTION

1) CELL DEATH	1
1.1) Apoptosis vs Necrosis: morphological features	1
1.2) THE APOPTOTIC MOLECULAR MACHINERY: From <i>Caenorabditis elegans</i> to humans	4
1.2.1) From CED-3 to caspases	4
1.2.2) From CED-4 to Apaf-1	8
1.2.3) From CED-9 to Bcl-2 family	10
1.3) DEATH RECEPTORS ACTIVATE THE APOPTOTIC MACHINERY	15
1.3.1) Signalling by CD95	16
1.3.2) Signalling by TNFR1	19
1.4) THE SPHINGOMYELIN CYCLE: AN INTRODUCTION	24
1.4.1) Enzymes in the sphingomyelin cycle	25
1.4.2) Ceramide and cell death	29
1.4.3) Death receptors and sphingomyelinases	32
1.5) NITRIC OXIDE	35
1.5.1) Nitric oxide: an historical perspective	35
1.5.2) Nitric oxide synthases: isolation and classification	36
1.5.3) NOS: catalytical mechanism	39

1.5.4)	NOS: sub-cellular localisation	42
1.5.5)	NOS: modulation of activity through phosphorylation	44
1.6)	NITRIC OXIDE AND CELL DEATH	45
1.6.1)	NO as a pro-apoptotic agent	45
1.6.2)	NO as an anti-apoptotic agent	49
1.7)	AIMS OF THE THESIS	52

Chapter 2: RESULTS

Effect of exogenous NO on early signalling by TNF- α in U937 cells

2.1)	Time course of TNF- α triggered sphingomyelinases activation	56
2.2)	NO inhibits sphingomyelinases activation	58
2.3)	Contribution of sphingomyelinases to early features of apoptosis	60
2.3a)	Specificity of sphingomyelinases inhibitors	60
2.3b)	Caspase-8 activation	63
2.3c)	DNA fragmentation	65
2.3d)	Phosphatidyl-serine exposure	68
2.4)	cGMP-dependent inhibition of acidic-sphingomyelinase accounts for apoptosis protection	70

Effect of endogenous NO on TNF- α induced cell death in HeLa/eNOS tet off cells

2.5)	Construction and characterization of HeLa/eNOS Tet-Off cell	72
2.5a)	Transfection and Western Blot	72
2.5b)	Sub-cellular localisation of eNOS	75

2.5c)	Transfected eNOS activity and cGMP assays	77
2.6)	eNOS expression modulates TNF- α induced cell death	81
2.7)	TNF- α stimulation increases eNOS activity and NO generation	85
2.8)	Role of sphingomyelinases in TNF- α -induced activation of eNOS	92

Chapter 3: DISCUSSION

3.1)	Exogenous NO and TNF- α signalling	97
3.2)	Endogenous NO and TNF- α signalling	101
3.3)	Cross talk between NO and ceramide: an hypothesis	106

Chapter 4: MATERIALS AND METHODS

4.1)	Materials	109
4.2)	Cell culture and treatments	110
4.3)	Measurement of sphingomyelinases activities in U937 cells	111
4.4)	Apoptosis detection in U937 cells	112
4.4a)	Western blot analyses of procaspase-8 and -3	112
4.4b)	Measurement of Caspase-8 activity	113
4.4c)	DNA fragmentation	113
4.4d)	Annexin-V staining	114
4.5)	Transfection and selection of HeLa cells conditionally expressing eNOS	114

4.6)	Western blot analysis in HeLa cells	115
4.7)	Immunofluorescence in HeLa cells	115
4.8)	Measurement of $[Ca^{2+}]_c$ in HeLa cells	116
4.9)	NOS Activity assays in HeLa Cells	116
4.10)	cGMP measurements in HeLa cells	117
4.11)	Apoptosis detection in HeLa cells	118
4.12)	Statistical analysis	118
REFERENCES		119
ACKNOWLEDGEMENTS		138
APPENDIX: Publications		139

ILLUSTRATIONS AND TABLES

Chapter 1) INTRODUCTION

Figure 1.1 Morphological features of apoptosis vs necrosis	3
Figure 1.2 Schematic model of caspase activation	7
Figure 1.3 Topology of Apaf-1 interacting proteins	9
Figure 1.4 <i>C.Elegans</i> model for Bcl-2 family proteins action	13
Figure 1.5 Evolutionary conservation of programmed cell death genes	14
Figure 1.6 Model for Fas triggered apoptosis	18
Figure 1.7 Model for TNF- α triggered apoptosis	21
Figure 1.8 A summary of apoptosome regulation	22
Figure 1.9 Sphingolipids structures and enzymes of metabolism	26
Figure 1.10 Proposed mechanism for TNF- α triggered activation of SMases	33
Figure 1.11 Relationship among the polypeptide sequence for NOS isoforms and cytochrome P450 reductase	38
Figure 1.12 Schematic representation of NOS reaction sequence	39
Figure 1.13 Central role of Calmodulin (CaM) in NOS activity	41
Figure 1.14 Signaling components characterizing NO-triggered apoptosis	48

Figure 1.15	
Effects of ceramide, NO and cGMP on TNF- α -induced TRADD recruitment to TNF-R1	53
Figure 1.16	
Effects of NO and cGMP on TNF- α -induced ceramide generation.	54
Fig. 1.17	
Effects of exogenous ceramide, NO, and cGMP on TNF- α -induced apoptosis.	55
 Chapter 2) RESULTS	
Figure 2.1	
Time course of activation of A-SMase (A) and N-SMase (B) by TNF- α in U937 cells	57
Figure 2.2	
NO inhibits TNF- α triggered A-SMase and N-SMase activities through the generation of cGMP and the activation of Protein Kinase G	59
Figure 2.3	
Specificity of action of drugs on A-SMase activity triggered by TNF- α	61
Figure 2.4	
Specificity of action of drugs on N-SMase activity triggered by TNF- α	62
Figure 2.5	
Involvement of A-SMase in caspase 8 activation triggered by TNF- α /CHX.	64
Figure 2.6	
Involvement of A-SMase in DNA fragmentation triggered by TNF- α /CHX	66
Table 2.1	
Inhibition by cGMP and potentiation by A-SMase of TNF- α -induced apoptosis are transient events	67
Figure 2.7	
Involvement of A-SMase in phosphatidyl-serine exposure triggered by TNF- α /CHX	69
Figure 2.8	
Inhibition of A-SMase by 8Br-cGMP accounts for its ability to reduce TNF- α /CHX triggered caspase-8 activation and DNA fragmentation	71
Figure 2.9	
Immunoblots of protein from HeLa tet-off cells.	74

Figure 2.10	
Double-staining immunofluorescence of HeLa tet-off cells	76
Figure 2.11	
Characterization of eNOS activity in HeLa tet-off cells	79
Figure 2.12	
cGMP generation in the HeLa/PC12 reporter system	80
Figure 2.13	
Effect of eNOS expression on TNF- α induced phosphatidyl-serine exposure	82
Figure 2.14	
Effect of eNOS expression on TNF- α induced hypodiploid DNA increase	83
Table 2.2	
effects of eNOS expression on the TNF- α -induced exposure of phosphatidyl-serine and formation of hypodiploid DNA	84
Figure 2.15	
Effect of TNF- α treatment on NO generation in HeLa/tet-off cells	86
Figure 2.16	
eNOS activation by TNF- α involves Pi3K activation.	87
Figure 2.17	
Different pathways of activation of eNOS	90
Figure 2.18	
Patterns of Akt (A) and eNOS (B) phosphorylation by different stimuli	91
Figure 2.19	
TNF- α activation of SMases in HeLa tet-off cells and specificity of SMase compounds	94
Figure 2.20	
TNF- α triggered phosphorylation of Akt and eNOS is downstream to N-SMase: Western Blot analysis.	95
Figure 2.21	
TNF- α triggered activation of eNOS is downstream to N-SMase: Arginine/Citrulline assay	96
Figure 2.22	
TNF- α triggered SMase activation is Pi3K independent	96

Chapter 3) DISCUSSION**Figure 3.1**

NO/cGMP dependent inhibition of SMases and of TNF- α triggered early apoptotic signalling **100**

Figure 3.2) TNF- α activates eNOS through a N-SMase/Pi3K/Akt dependent signalling pathway **105**

Figure 3.3

Hypothetical model of cross-talk between NO and SMase in regulation of TNF- α triggered apoptosis **108**

ABSTRACT

Tumour Necrosis Factor- α (TNF- α) triggers cell death via apoptosis through a well defined signaling pathway activated by its p55 kDa receptor. The problem of the modulation of this signaling by intracellular second messenger is, on the contrary, a very debated and open field. In a previous paper (**De Nadai et al, 2000**) we have shown that the lipidic messenger ceramide is able to increase recruitment of the TRADD protein to the p55 kDa receptor, caspase-8 activation and thus apoptosis. In the same paper we have shown that nitric oxide was able to inhibit these same features but the mechanisms through which nitric oxide and ceramide interact, however, are unclear.

I have studied the contribution of ceramide-generating enzymes, i.e. the acidic and neutral sphingomyelinases (A- and N-SMase), to the apoptotic response to TNF- α , as well as their cross-talk with NO in two different cellular systems, the pro-monocytic cell line U937 and HeLa cells stably transfected with bovine eNOS cDNA under the control of a tetracycline repressed promoter (HeLa-eNOS tet-off cell line).

In U937 cells, TNF- α rapidly triggered both A- and N-SMase activity. Cells pre-incubation with the NO donor SNAP inhibited activation of both enzymes. These effects of the NO donor were mimicked by 8-Br cyclic GMP, and reverted by the guanylate cyclase inhibitor ODQ. A-SMase inhibitors reduced TNF- α triggered caspase-8 activation, while inhibition of N-SMase resulted in no effect. In HeLa cells eNOS expression was found to exert a protective effect on TNF- α induced apoptosis and, moreover, TNF- α was found to activate eNOS through a N-SMase/Pi3K/Akt dependent signalling pathway.

These results suggest the existence of a regulatory loop in which NO and ceramide acts in concert in order to modulate TNF- α signalling.

Chapter 1

INTRODUCTION

1) CELL DEATH

Programmed cell death (apoptosis) is an ubiquitous process that plays a fundamental role both in physiology and pathology: it is a key player both during embryological morphogenesis as well as in adult stages of the organism, where it regulates crucial events, e.g. tissue turnover and immune response, thereby contributing to the homeostasis of the organism (**Raff, 1998**). Its dis-regulation may cause or contribute to the onset of various pathological states, such as neuro-degenerative diseases (e.g. Alzheimer disease) or cancer (**Honing and Rosenberg, 2000; Cory and Adams 2002**).

The first experimental definition of apoptosis took origin from observation carried out during experiments of liver degeneration: Kerr and collaborators induced liver atrophy in the rat by tying off a large branch of the portal vein and described a discrete demise of cells characterized by a sequence of changes defined first as shrinkage necrosis and then as apoptosis (**Kerr et al, 1972**). This and a number of subsequent critical observations opened the way to an enormous increase in interest regarding apoptosis, with the definition of both its morphological and biochemical features, especially in opposition to the other known form of cell demise, i.e. necrosis.

1.1) Apoptosis vs Necrosis: morphological features

The most important feature of apoptosis is represented by the stereotyped sequence of morphological and biochemical events that literally make the cell disappear upon the death stimulus. These events can be triggered by either an internal clock or by extracellular factors such as hormones, cytokines and other chemical and physical agents.

Briefly, the cell starts to lose volume rapidly, detaching from its social environment and profoundly modifying its plasma membrane characteristics, with the exposure of components normally hidden or little expressed, important for dying cell recognition by macrophages. The internal organization of the cell is maintained, at least in the initial

phases of the process, while the most evident events occurs at the nuclear level: the nucleolus and basal lamina disgregate, chromatin condenses and is cut in 200 bp (or multiple of this) fragments, representing the length of the DNA inter-nucleosomic traits. Discrete chromatin fragments thus formed move toward the plasma membrane, where they are engulfed by the membrane itself to form apparently discrete structures: these events give rise to blebbed shape of the cell as observed by both light and electron microscopy. Blebs detach from the cell body, engulfing part of the cytoplasm and of the nuclear material, and form the so-called 'apoptotic bodies" recognized and phagocytosed by bystander cells. A particular feature of this process is that it happens without spilling of cytosolic content to the extracellular milieu, and without causing any inflammatory reaction (**Fig 1.1 A and C**).

All this process is very different from necrosis, which represents a passive process, the consequence of gross injuries to the cell. When a cell dies by necrosis early changes can be identified at the level of the plasma membrane which shows progressive discontinuities, causing abrupt swelling and organelle disruption. (**Fig 1 B and D**). The cytoplasm and the plasma membrane are the first targets of the necrotic process, while the nucleus, at variance with what is observed during apoptosis, appears relatively preserved.

When cultured cells are analysed by light or fluorescence microscopy, apoptotic cells have a distinct appearance from necrotic cells with differences that mainly concerns shape and structure: the final stage of apoptosis is cell splitting in numerous fragments, whose most common final fate in the organism is to be engulfed by phagocytes. In contrast, no cell fragmentation appears in the course of necrosis. Rather what happens is a general cell hydration process, followed by cell disruption. Electron microscopy provides a detailed characterization of both phenomena, as illustrated in **Fig 1.1**.

A very simplified, but useful view that we could have from this brief morphological description of the two different type of cell death is that necrosis represents a passive response to an insult, with the cell literally exploding in an uncontrolled way, while apoptosis represent an active response to death stimuli activating a complex machinery strictly controlled by an evolutionary conserved genetic background. Such machinery will be described in the next section

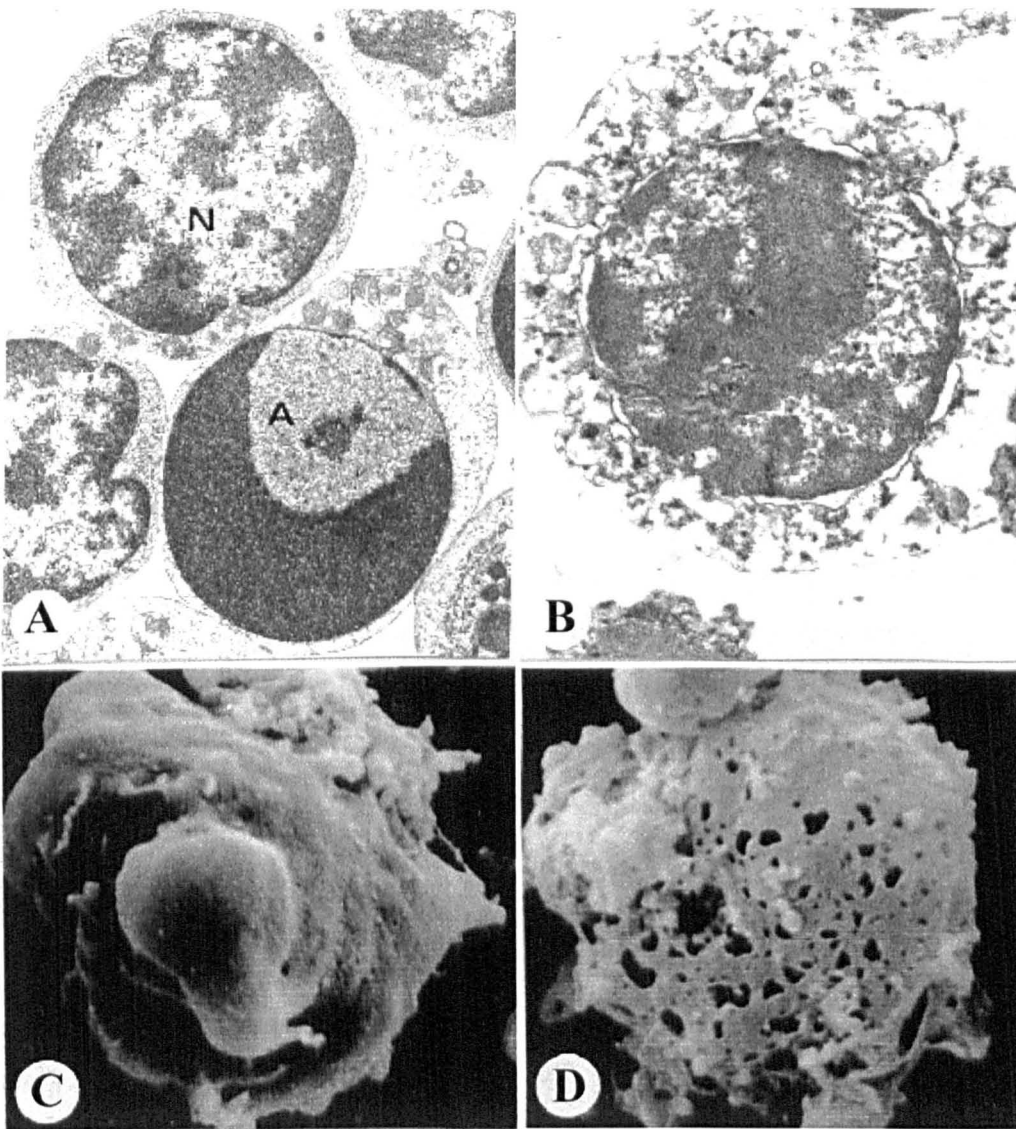


Figure 1.1) Morphological features of apoptosis vs necrosis

- A)** Transmission Electron Microscope (TEM) image of an apoptotic cell (A) compared with a normal cell (N): Chromatin packaging clearly appears in A, strongly different from its normal arrangement. The good preservation of membrane and organelles is also different (magnification: x 8000)
- B)** TEM image of a necrotic cell: the disruption of plasma membrane and organelles is clearly observable, while the nucleus is retaining its normal morphology (magnification: x 8000)
- C)** Scanning Electron Microscopy (SEM) image of an apoptotic cell: the plasma membrane appears homogeneously preserved, while it is clearly visible surface blebbing (magnification: x5000)
- D)** SEM image of a necrotic cell: The plasma membrane shows numerous lesions (magnification: x5000)

1.2) THE APOPTOTIC MOLECULAR MACHINERY: From *Caenorhabditis elegans* to humans

Most of the knowledge about the basal mechanism responsible of programmed cell death derives from the genetic analyses carried out in the nematode worm *Caenorhabditis elegans*. Onset and progress of development in this animal is very well defined, with 1090 cells that constitutes the adult body and 131 cells that die in the course of development. The unraveling of this sophisticated plan was important because it permitted to easily identify and characterise all mutants bearing abnormalities in cell number and survival upon development of the whole organism, and thus to define the role of each gene and its product in the apoptotic process.

Caenorhabditis mutants aberrant in the death genetic program depend on in three principal genes, the so-called Cell Death genes (*ced* genes), whose products operate the regulation of cell death. CED-3 and CED-4 act to kill cells, while CED-9 suppresses killer genes action (Hengartner et al, 1994).

CED-3 has been surprisingly identified as a proteolytic enzyme, a protease (Yuan et al, 1993), CED-4 was found to act as a positive regulator of CED-3, being able to bind it and to promote its proteolytic activity. In contrast CED-9 was identified as a CED-3 negative regulator, since it is able to bind CED-4, thus preventing the activation of CED-3 (Chinnaiyan et al, 1997). What happens normally is that CED-9 is complexed with CED-3 and CED-4 keeping CED-3 inactive. The apoptotic stimuli cause dissociation of CED-9, allowing the activation of the CED-3 protease and thereby committing the cell to die by apoptosis.

This simplified mechanism represents the prototypic model from which the much more complex cell death machinery of the higher organisms has emerged, as described below.

1.2.1) From CED-3 to caspases

The protein encoded by *ced-3* gene is very similar to the human interleukin-1-converting enzyme (ICE) protein (Yuan et al, 1993; Thornberry et al, 1998), an intracellular protease that cleaves interleukin-1, a signalling protein inducing inflammation, out of a larger precursor protein. Recognition of the similarity between CED-3 and ICE proteins was actually the first indication that the apoptotic programme is dependent on proteolysis. Very soon, starting from this similarity, new members of the CED-3/ICE family of proteases

were identified, many of which become activated during apoptosis (**Nicholson et al, 1997**). These proteases share two conserved features: all of them are cysteine proteases containing a conserved QACXG penta-peptide, including the active-site cysteine (**Cohen 1997**); and all of these enzymes have a unique strong preference for cleaving peptide bonds C-terminal to aspartate residues. For these reasons, these enzymes have been collectively called cysteine-aspartate proteases, caspases (**Alnemri et al, 1996**). Up to date, the caspase family of proteases consists of at least 14 mammalian members that are constitutively expressed in almost all cell types as inactive proenzymes (zymogens) that became processed and activated in response to a variety of pro-apoptotic stimuli.

Pro-caspases (32-56 kDa) contain four domains: an N-terminal prodomain, a large subunit (17-21 kDa), a small subunit (10-13 kDa) and a short linker between the large and small subunits (**Figure 1.2; Earnshaw et al, 1999**). Caspase activation involves a proteolytic processing of the proenzyme at specific aspartate residues between domains, resulting in removal of the prodomain as well as the linker region, with formation of a heterodimer containing a large and a small subunit (**Liang and Fesik, 1997**). The active caspase is a tetramer composed by two such heterodimers.

The importance of caspases for the apoptotic process has been clearly demonstrated by several independent findings, among which:

- i) Over-expression of caspases has a severe cytotoxic effect (**Chang and Yuang, 2000**)
- ii) Caspase inhibitors are able to effectively inhibit apoptosis induced by various stimuli (**Ekert et al, 1999**)
- iii) Knock-out animal models lacking caspases shows profound defects in apoptosis (**Zheng and Flavell, 2000**)

Caspases differ in their substrate specificity and can be divided into three subgroups, based on preferred tetra-peptide sequences after which active enzymes cleave their respective substrates. The P4 site (that means 4 aminoacids N-terminal to the cleavage site) is the most critical determinant of substrate specificity.

Caspases of group I (caspase-1, -4 and -5) cleave preferentially after the WEHD motif, group II (Caspase-3 and -7) after DEXD, whereas the optimal cleavage sequences for caspases belonging to group III (caspase-6, -8, -9 and -10) are the (I/L/V) EXD motifs (**Thornberry et al, 1997**). Only caspase-2 cleaves preferentially after the penta-peptide sequence VDVAD; the cleaving sites for caspases-11, -12, -13 and -14 are not yet known.

Evidence for the sequential activation of caspases has led to the concept of a caspase cascade (**Hirata et al, 1998**). During apoptosis, initiator caspases are thought to be initially auto-catalytically activated in response to apoptogenic stimuli. Subsequently, they transmit the signal by cleaving, and thereby activating, downstream effector caspases that proceed to the cleavage of other specific proteins in order to 'dismantle' the cell (**Salvesen and Dixit, 1999**).

Among caspase substrates are cytoskeletal proteins, such as lamin and actin, cytoskeletal regulators, such as focal adhesion kinase (FAK; **Wen et al, 1997**) or gelsolin (**Kothakota et al, 1997**), and proteins involved in DNA repair and cell cycle regulation, such as polyADP-ribose polymerase (PARP) or the retinoblastoma (Rb) protein, respectively (**Chang and Yang, 2000**). Caspases also activate a DNA-cleaving enzyme (a DNase) by cleavage of an inhibitory subunit enabling the enzyme (called CAD for caspase-activated DNase) to cut DNA in the nucleus (**Enari et al 1998**). A survey of these caspase substrates indicates that caspases participate to the apoptotic cell death programme in a very coordinated manner which includes the following sequence of events: cut off of contacts with surrounding cells, reorganization the cytoskeleton, shut down of DNA function and structure, and finally disgregation of the cell into apoptotic bodies, that are engulfed by macrophages and bystander cells.

Most experimental models of apoptosis involve the activation of caspases, and until now no activation of caspases has been found in the context of necrotic cell death (**Armstrong et al, 1997**); yet, apoptosis may occur in the absence of caspase activation. Because of this, detection of caspase activation in cells is considered as a good discriminating criterion to distinguish the two types of cell death, concerning at least the early features of cell death.

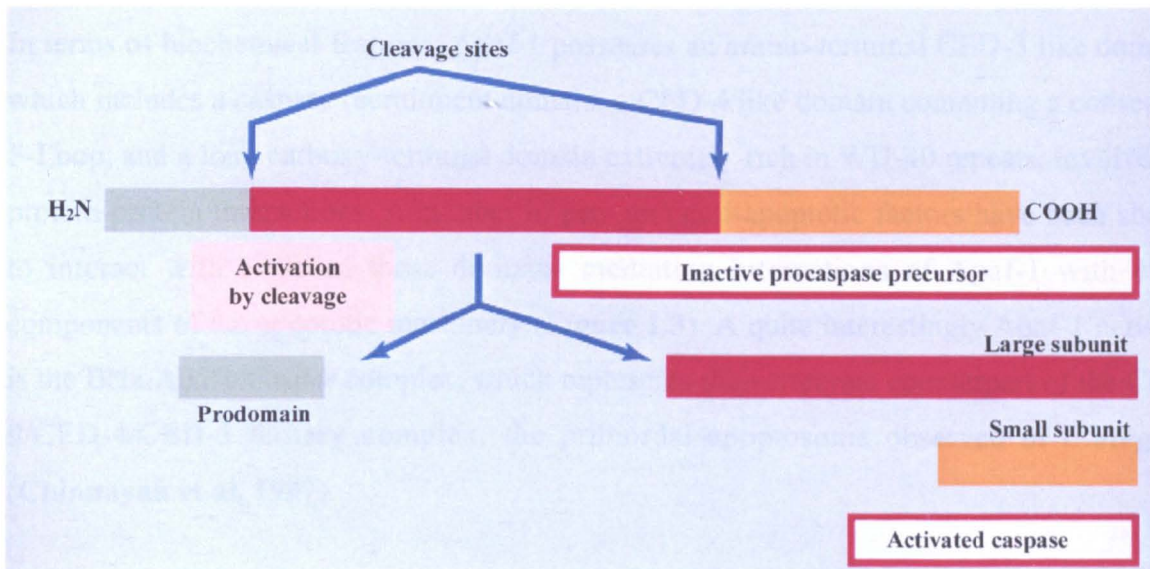


Figure 1.2) Schematic model of caspase activation

The whole protein is constituted by a large inactive precursor, the pro-caspase, which is activated following a proteolytic cleavage at two particular aspartic acid residues, with removal of the prodomain and formation of the large and small subunits. These subunit forms a heterodimeric complex to form the complete active enzyme. There are evidences that a complete enzyme is constituted by a complex made up from two large and two small subunits (not shown)

Adapted from (**Raff, 1998**)

1.2.2) From CED-4 to Apaf-1

The human CED-4 homologue has been identified by Zou and collaborators (**Zou et al, 1997**) by an in vitro reconstitution approach, and has been termed apoptotic protease activating factor (Apaf-1). It has been shown to participate in the cytochrome c (cyt-c)/dATP-dependent activation of caspase-3 through the proteolytic activation of caspase-9 (**Li et al, 1997**).

In terms of biochemical features, Apaf-1 possesses an amino-terminal CED-3 like domain, which includes a caspase recruitment domain, a CED-4 like domain containing a conserved P-Loop, and a long carboxy-terminal domain extremely rich in WD-40 repeats, involved in protein-protein interactions. A number of pro- and anti-apoptotic factors have been shown to interact with some of these domains mediating interactions of Apaf-1 with other components of the apoptotic machinery (**Figure 1.3**). A quite interestingly Apaf-1 network is the Bclx/Apaf1/Casp-9 complex, which represents the vertebrate counterpart of the CED-9/CED-4/CED-3 ternary complex, the primordial apoptosome observed in *C.elegans* (**Chinnayan et al, 1997**).

Apaf-1 is an important player in the activation of caspase-9 in a pathway that involves cyt-c. The latter is required for the formation of a complex, sufficient to activate caspases even in cell-free extracts (**Liu et al, 1996**). In vitro binding of Apaf-1, pro-caspase-9 and cyt-c, in the presence of ATP, results in the activation of caspase-9. However the exact target site of cyt-c in Apaf-1, as well as its action, has not yet been precisely defined. Currently, because of its homology to CED-4, which has ATPase-like functions (**Aravind et al, 1999**), Apaf-1 is considered to be an ATPase, crucial to its role in caspase activation (**Chaudary et al, 1998**). Conversely, a truncated form of Apaf-1, lacking the WD-40 domain, is able to promote caspase-9 activation in the absence of cyt-c or dATP (**Srinivasula et al, 1998**). Taken together, these observation indicate that cyt-c, dATP and the WD-40 domain are involved in the conformational changes of Apaf-1 that are necessary to induce caspase-9 activation

The observations summarized above provide evidence for an evolutionary conservation among *C.elegans* and vertebrates of the basal apoptotic machinery at a biochemical and cellular level. The importance of apoptosis in development grows with the complexity of the organism. Although with nearly 20% more cells, apoptosis deficient worms can have a normal life span. By contrast, the various caspase KO mice all die because of several

abnormalities resulting from apoptosis deficiency (**Zheng and Flavell, 2000**). The Apaf-1 deficient mice follow this rule, having a massive developmental phenotype: they die between embryonic day 16 and post-natal day 0, exhibiting reduced apoptosis in the brain and dramatic cranio-facial alterations (**Cecconi et al, 1998**).

These results strongly indicate that Apaf-1 and caspase 9 are components of the same apoptotic pathway, resembling the *C.elegans* situation, where CED-4 was able to activate CED-3.

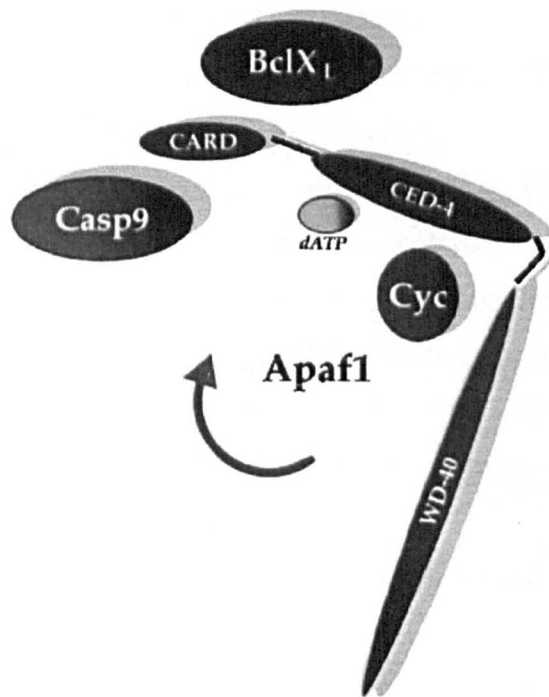


Figure 1.3) Topology of Apaf-1 interacting proteins

Apaf-1 protein is composed of three domains, an N-terminal caspase recruitment domain (CARD), an Ap-ATPase domain (CED-4 like) and a WD-40 repeats domain (WD-40) for a molecular weight of 135 kDa. The apoptosome components are depicted in close proximity of their potential interaction site with Apaf-1 domains. dATP is an essential cofactor for Apaf-1 activity. The arrow indicates the potential of APAF-1 to get its NH2 and COOH terminus into contact, with different functional implications.

Adapted from (**Cecconi, 1999**)

1.2.3) From CED-9 to Bcl-2 family

As previously seen, the genetic analysis of the nematode *C.elegans* revealed two loci, *ced-3* and *ced-4*, essential for programmed cell death during development. The third *ced-9*, was found to be a regulator of their action. If *ced-9* is inactivated, most of the cells in the developing worm die. Quite remarkably, however, if also *ced-3* or *ced-4* are inactivated, the worm and all of its cells remain alive (Adams et al, 1998). CED-9 appears so to represent a true regulator of the apoptotic machinery. The first mammalian regulator emerged when *bcl-2*, a gene activated by chromosome translocation in human follicular lymphoma, was unexpectedly found to permit survival in a quiescent state of cytokine-dependent hematopoietic cells, in the absence of cytokine (Vaux et al, 1988).

Bcl-2 and CED-9 are functional and structural homologues. The similarity is so strong that when the human *bcl-2* gene is artificially introduced in the worm it can functionally substitute for CED-9, thus inhibiting apoptosis (Vaux et al, 1992). The discovery of Bcl-2 has fostered identification of a large family of proteins with different, and sometimes counter-acting functions. At least 15 protein members of the family have been identified up to date in mammalian cells, falling into three distinct groups. Bcl-2 and several close relatives are able to exert apoptosis inhibition (Vaux et al, 1988), whereas structural similar relatives such as Bax (Gross et al, 1999) and distinct cousins such as Bik and Bim instead fosters death (Kelekar and Thompson, 1998).

The various subfamilies share sequence similarities: Bcl-2 and Bax subfamilies share three of four conserved Bcl-2 homology sequence motifs (BH motifs), and can assume a similar conformation (Sattler et al, 1997). In contrast, members of the third subfamily share only one domain (BH3) and are unrelated even to one another. Most members of the wider clan possess a hydrophobic C-terminal segment, which facilitates their interaction with the endoplasmic reticulum, nuclear envelope and outer mitochondrial membranes, where the pro-survival members normally reside, and where they assemble during apoptosis.

Members of the pro- and anti-apoptotic families are able to heterodimerize: the BH3 helix of the apoptogenic proteins can bind to a hydrophobic groove in the pro-survival proteins, created by α -helices in the BH3, BH1 and BH2 regions. Thus BH3-only proteins can serve as ligands that lock a pro-survival protein into an inactive conformation. In essence: interactions between two proteins with opposite roles result in inhibition of each other function. The ratio of suppressor-to-promoter determines the cell susceptibility to apoptosis.

Genetic studies indicate that without a Bcl-2 type guardian, most cells of the metazoans are primed to death: in *C.Elegans*, the *ced-9* gene is required for embryogenesis, apparently because the lack of CED-9 permits activation of CED-3 by CED-4 (**Horvitz, 1999**). Disruption of the mammalian pro-survival genes, by contrast, produces phenotypes only in specific tissues (**Adams and Cory, 1998**), presumably due to their redundancy and partially overlapping expression patterns.

The exact biochemical mechanism by which the pro-survival Bcl-2 family members exert their function is still debated. For example, it has not been clarified whether these proteins keep the caspase activators in check directly or indirectly: results from the *C.elegans* model, moreover, favour a direct sequestration model as shown in **figure 1.4**. In this model CED-4 co-localizes with CED-9 on mitochondria until a death signal induces the displacement of CED-4, which then activates CED-3 (**Chen et al, 2000**). It therefore seems plausible that CED mammalian homologous also associate.

Bcl-xl was initially reported to interact with Apaf-1; further studies did not provide evidence for any such complexes (**Moriishi et al, 1999**) and supported the conclusion that Apaf-1 is a monomeric cytosolic protein (**Haussman et al, 2000**). Nevertheless, CED-9 and its mammalian homologues must act similarly, because a *bcl-2* transgene can rescue cells in CED-9 deficient nematodes (**Hengarter and Horvitz, 1994**).

Alternative models suggesting an indirect mode of function for Bcl-2-like proteins are recently gaining momentum. In particular it has been proposed that Bcl-2 governs caspase activation indirectly through effects on organelles, in particular mitochondria. Bcl-2 over-expression precludes all mitochondrial disturbances associated with apoptosis, such as pH, membrane permeability and outer membrane integrity (**Green and Reed, 1998**). The mitochondrial inter-membrane space contains several apoptogenic proteins and their release might represent the most important death signal. Cyt-c has been the first recognized apoptogenic signal escaping from the mitochondria. It represents therefore a critical co-factor (together with ATP) for the activation of caspase-9 by Apaf-1 (**Liu et al, 1996**).

Data emerging from the identification of the tri-dimensional structure of a Bcl-2 family member may contribute to give an answer to the question of how Bcl-2 are able to affect mitochondria: the resemblance of the 3D structure of Bcl-xl to that of membrane penetrating bacterial toxins lead to the hypothesis that all family members possessing hydrophobic BH1-BH2 domains might form pores in organelle membranes (**Kelekar and Thomson, 1998**). This feature has been shown with lysosomes. Whether this occurs in live cells remains to be established. While it is easy to explain how the pore-forming activity

leads to the cytotoxic effects of Bax-like proteins, it is more difficult to foresee how pores formed by Bcl-2-like molecules might protect cells.

Rather than forming new pores in the mitochondria, Bcl-2 family members might stabilize the pre-existing channels through which adenine nucleotides and other small molecules traffic occurs, i.e. the 'permeability transition pore', that is believed to form across sites of contact between the inner and outer membranes (**Crompton, 1999**). The open channel allows passage of molecules up to 1500 daltons and the pore in the outer as well as in the inner membrane appears to be gated (**Vander Heiden, 2000**). Opening of the inner membrane channel can dissipate the H^+ gradient across the membrane, uncoupling the respiratory chain from ATP production. The resulting matrix swelling was proposed to rupture the outer membrane (**Vander Heiden et al, 1997**), but neither swelling nor rupture has been clearly shown to be common feature of apoptosis (**Von Ahsen et al, 2000**). Moreover, evidence of permeability transition pore activation is still lacking.

Despite the amount of studies regarding the Bcl-2 family of proteins the whole picture of interactions between the various family members is not known in detail, and many questions remain open. Much remains to be understood about the role of BH3 containing proteins, and about how damage signals are sensed. For Bax-like proteins, the questions regard the mode of activation, whether they are activated by BH3-only ligands such as Bid; whether they represent a trigger or an amplification mechanism for apoptosis. In addition it is not yet clear whether they mediate death primarily by compromising their pro-survival relatives, by activating caspases independently or by disruption of mitochondrial function. For the pro-survival proteins, open issues are how the heterodimerization is able to alter them, how their function in the ER and nuclear envelope affect the mitochondria and the nature of their immediate downstream effectors.

A recent progress in the ER field has demonstrated that in cells over-expressing Bcl-2 the cistaernae content of Ca^{2+} is significantly reduced (**Marsden et al, 2002**). On activation of Ca^{2+} release, therefore influx is smaller and this protects mitochondria from Ca^{2+} damage. Although the mechanism of Bcl-2 action on ER membranes is not yet clear (leakage?) this ER process appears to play a relevant role in the effect of the oncogene.

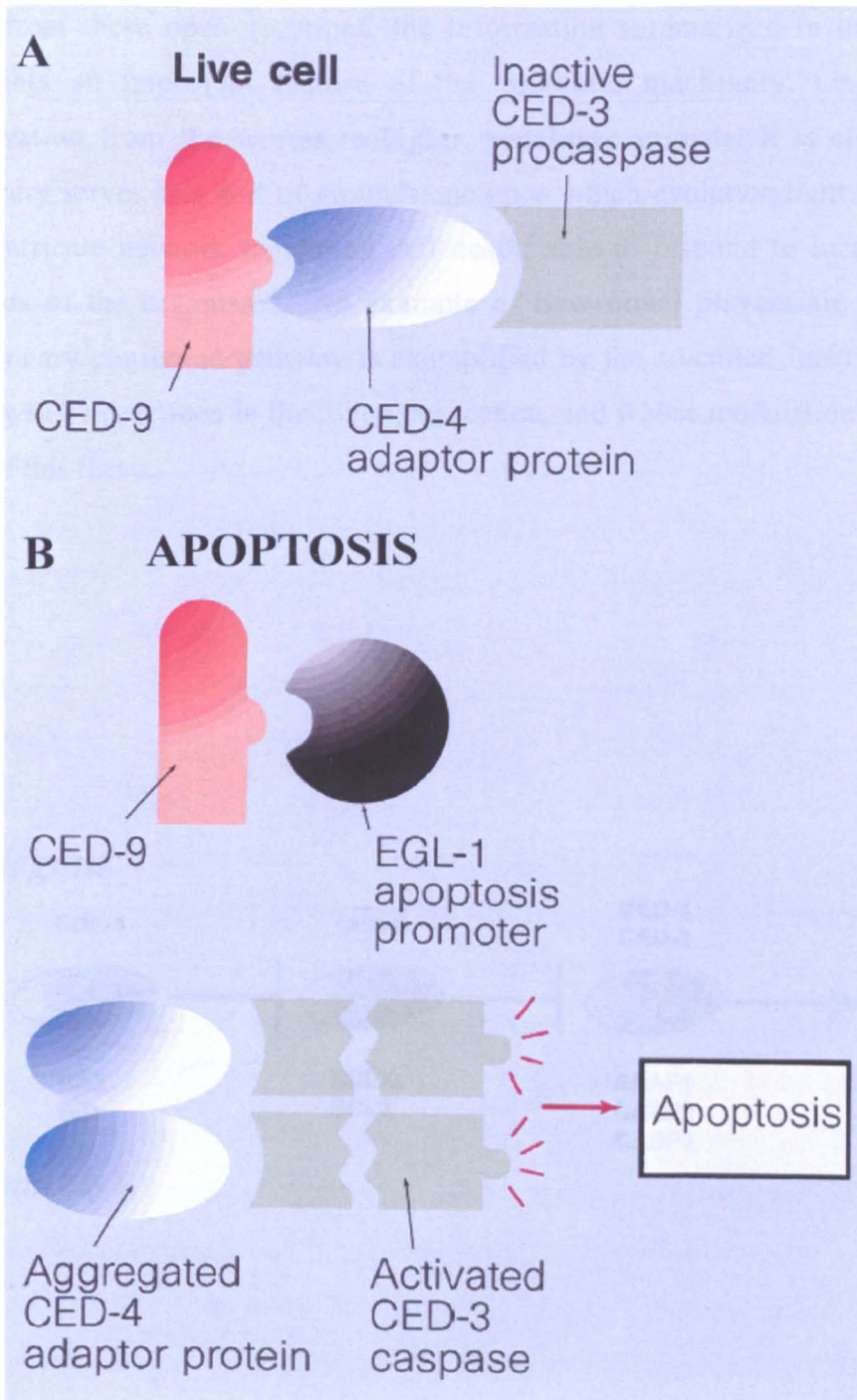


Figure 1.4) *C.Elegans* model for Bcl-2 family proteins action

- A)** In living cells, the CED-9 protein forms a complex with CED-4, inhibiting it from aggregating and interacting with the CED-3 pro-caspase.
- B)** In dying cells, an apoptosis-promoter of the Bcl-2 family (here is EGL-1 depicted) binds to CED-9, releasing it from the complex with CED-4 and CED-3. CED-4 is so able to aggregate, leading the bound CED-3 molecules close enough to exert their auto-activation, following the proximity activation model .
- In the context of this model the various complexes are probably bound to intracellular membranes by CED-9.

Adapted from (Raff, 1998)

Apart from these open questions, the information summarized in this chapter clearly highlights an important feature of the apoptotic machinery, i.e. its evolutionary conservation from the worms to higher vertebrate animals. It is clear that the basal machinery serves as a sort of groundstone upon which evolution built up a progressively more intricate network regulating cell death, able to respond to increasingly complex demands of the organisms.. An example of how novel players are integrated in this evolutionary conserved pathway is exemplified by the so-called 'instructive' apoptosis, which will be described in the following section, and whose modulation will be the central point of this thesis.

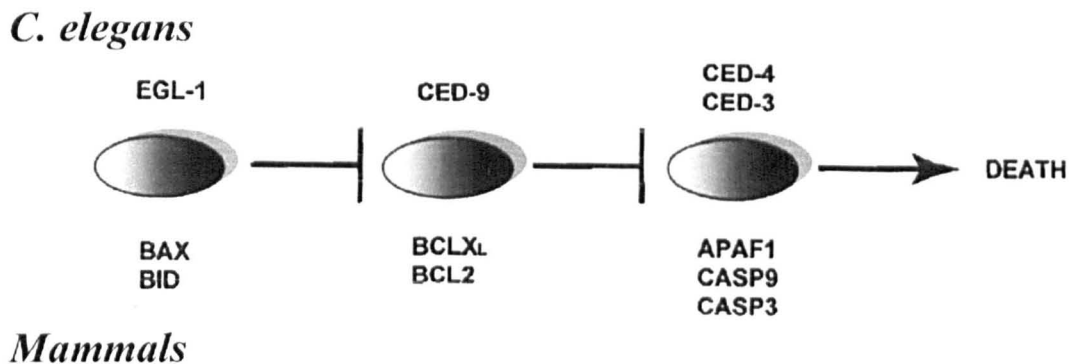


Figure 1.5) Evolutionary conservation of programmed cell death genes

This diagram schematically represents the conservation of the apoptotic genetic program. The upper diagram shows the order of function of *ced* genes in *C.elegans* while the lower diagram shows the conserved mechanisms in mammals, where due to the complexity of the organisms, entire gene families play the counterpart roles of *C.elegans* proteins.

Adapted from (Cecconi, 1999)

1.3) DEATH RECEPTORS ACTIVATE THE APOPTOTIC MACHINERY

Cells are able to integrate signals coming from either the environment or from the intracellular milieu in order to keep their survival machinery in check: losing contacts from the surroundings or an internal damage (such as DNA damage) trigger the initiation of apoptosis (**Evan et al, 1998**). Remarkably, mammals have also evolved another mechanism to actually direct individual cells to death, which is based on a receptorial protein system, the so-called Death Receptors. These receptors play a key role in instructive apoptosis, which is particularly important in the immune system (**Osborne, 1996**), because they are able to connect extracellular signals to the apoptotic machinery. As a whole the process stems evolutionary from the one described in the previous section. This is a nice example of how nature is able to integrate pre-existing signalling modules with novel emerging necessity, in order to provide appropriate answers to novel biological demands.

Death receptors belong to the tumor necrosis factor (TNF) receptor gene superfamily, defined by the presence of conserved domains in the proteins they code for. These proteins contain from 2 to 6 repeats of cysteine-rich extracellular sub-domains, which represent also the portion of the each protein sharing the highest similarity with the other family members; in addition, they contain a homologous sequence which codes for an approximately 80 aminoacid domain near the carboxy-terminus of the protein, which has been named the death domain (DD; **Tartaglia et al, 1993**) and which has the tendency to self-aggregate. The DDs act as modules mediating ligand-dependent recruitment of other DD-containing proteins. The latter are crucial to propagate the signal from outside to inside the cell and have an obligate role in inducing cyto-toxicity, enabling receptors to engage the cell's apoptotic machinery, once they are activated.

The tertiary structure of the death domains has been resolved by heteronuclear multidimensional Nuclear Magnetic Resonance (NMR) spectroscopy, showing that it includes a protein fold consisting of six antiparallel, amphipathic α helices (**Huang et al, 1996**). Many charged amino acids are present on the surface of this structure and this is probably the structural reason responsible for mediating the interaction between various DDs. DDs have been conserved through metazoan evolution: they share limited homology with the protein product of the *Drosophila reaper* gene, originally identified by deletion mapping studies as a gene essential for many cell deaths occurring during normal *Drosophila*

embryogenesis (Golstein et al, 1995). Deletion of *reaper* not only blocks developmental cell death but also suppresses cell death in response to X-ray-irradiation.

Death receptors compose a large family, with the best-characterized examples being CD95 (also named Fas or APO-1) and the p55 KDa receptor for TNF- α (TNF-R1). The signal transduction of these two receptors will be briefly described below.

1.3.1) Signalling by CD95

CD95 and its ligand, CD95L (or Fas ligand) plays an important role mainly in three types of physiologic apoptosis:

- i) peripheral deletion of activated mature T cells at the end of the immune response.
- ii) killing of targets such as virus-infected cells or cancer cells by cytotoxic T-cells.
- iii) killing of inflammatory cells at immunoprivileged sites such as the eye.

The biological importance of CD95 has been determined from mouse KO as well as from human pathologies in which expression of inactive defective genes for CD95 or CD95L leads to accumulation of peripheral lymphoid cell and to a fatal autoimmune disease characterized by massive enlargements of lymphnodes (Nagata, 1997).

CD95 is a type-I membrane protein with a homotrimeric structure: its ligand has a trimeric structure and each CD95L trimer binds to three molecules of the receptor. Since death domains have the tendency to associate one with the others, the binding of CD95 triggers the clustering of receptor's death domain, a finding supported by nuclear magnetic resonance structure analysis and mutagenesis studies (Huang et al, 1996).

An interacting protein, the Fas-Associated Death Domain (FADD; named also Mort1), was identified with the yeast two-hybrid system, using the Fas cytoplasmic region as a bait. This protein, through its own death domain and following the conformational change of the activated receptor, is able to bind to clustered receptor death domain (Chinnaiyan et al, 1995).

FADD N-terminal region contains also another important domain, the Death Effector Domain (DED), responsible for executing downstream signal transduction. In fact, DED represents an example of a more global homophilic interaction domain termed CARD (as for Caspase Recruitment Domain) found in several caspases and able to bind to a similar domain present in a repeated fashion within the zymogen form of caspase-8 (also called FLICE, or MACH) (**Boldin et al, 1996**). Following recruitment operated by FADD, pro-caspase-8 activates itself through self-cleavage (**Muzio et al, 1998**) and triggers the activation of downstream effector caspases, such as caspase-9 (which is the mammalian homologue of the nematode protein CED-3) resulting in an amplification effect that commits the cell to apoptosis (see **Figure 1.6** for a schematic model of CD95 signalling). The key role played by FADD in cell death signal have been confirmed by the FADD gene KO mice (**Zhang et al, 1998**) and by transgenic mice expressing in T-cells a dominant negative mutant of FADD (**Newton et al, 1998**). Both these studies demonstrate the obligate role of FADD in the CD95 triggered cell death.

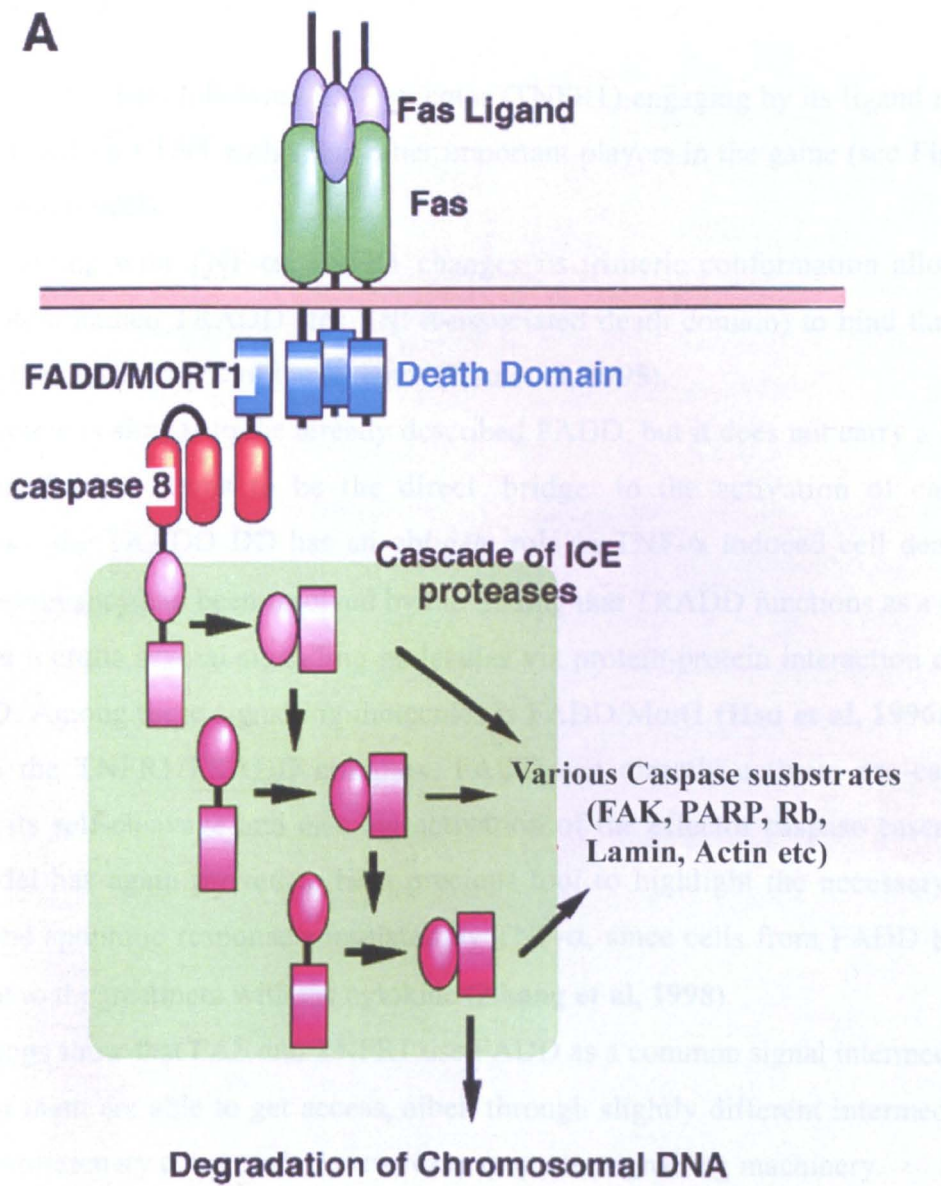


Figure 1.6) Model for Fas triggered apoptosis

Binding of Fas ligand to its receptor induces a tightening of its structure, with a concomitant conformational change, which cause the recruitment of caspase-8, via the adaptor protein FADD/MORT1. The oligomerization of caspase-8 results in its self-activation with the activation of the ICE protease cascade. The activated downstream caspases can cleave various substrates, such as Poly(ADP)ribose polymerase (PARP), focal adhesion kinase (FAK), lamin, actin and various others causing the morphological changes of the cell and leading to its apoptotic demise

Adapted from (Nagata, 1997)

1.3.2) Signalling by TNFR1

The sequence of events following TNF receptor (TNFR1) engaging by its ligand is similar to that described for CD95 with some other important players in the game (see **Figure 1.7** for a schematic model).

Upon the binding with TNF- α , TNFR1 changes its trimeric conformation allowing an adaptor protein named TRADD (for TNFR-associated death domain) to bind through its own DD to the clustered DD of the receptor (**Hsu et al, 1995**).

TRADD protein is similar to the already described FADD, but it does not carry a DED, so this protein doesn't seem to be the direct bridge to the activation of caspases. Nevertheless, the TRADD DD has an obligate role in TNF- α induced cell death. This apparent discrepancy had been resolved by the finding that TRADD functions as a platform adapter that recruits several signalling molecules via protein-protein interaction domains, such as DD. Among these signalling molecules is FADD/Mort1 (**Hsu et al, 1996a**). Once coupled to the TNFR1/TRADD complex, FADD can directly activate pro-caspase-8, promoting its self-cleavage and ensuing activation of the effector caspase cascade. The mouse model has again proved to be a precious tool to highlight the necessary role of FADD in the apoptotic response stimulated by TNF- α , since cells from FADD KO mice are resistant to the treatment with the cytokine (**Zhang et al, 1998**).

These findings show that FAS and TNFR1 use FADD as a common signal intermediate and that both of them are able to get access, albeit through slightly different intermediates, to the same, evolutionary conserved, downstream apoptotic signalling machinery.

Unlike CD95, whose signal transduction seems to be restricted to activation of pro-apoptosis proteins, TNFR1 is also able to recruit other proteins such as RIP and TRAF, always via TRADD intermediation, which activate not only apoptotic but also protective pathways.

RIP protein is a serine/threonine kinase that is able to preferentially bind TRADD via a DD (**Hsu et al, 1996b**). This protein may trigger a second apoptotic pathway starting from TNFR1: when over-expressed RIP is able to induce apoptosis, but its signal transduction is not entirely clear. The RIP DD is indeed responsible for the propagation of the death signal, while the kinase domain has no apparent role. Moreover RIP doesn't possess a DED, and this suggests that it recruits some other effector proteins, still unknown. A likely candidate, RAIDD (RIP-associated Ich1/CED-3 homologous to a Death Domain), has been identified. RAIDD is able to interact with RIP through a DD and to recruit caspase2 to RIP (**Duan**

and Dixit, 1997). However its involvement in TNF-R1 triggered apoptosis has not yet been clarified.

RIP is also able to function as a scaffolding protein for the binding of members of the TRAF (TNFR-Associated Factor) family (**Rothe et al, 1994**). Activation of these proteins is crucial for the switching on of the signalling pathway leading to the Nf-kB, a key transcription factor, which has a crucial role in triggering the expression of anti-apoptotic proteins. Blockade of NF-kB activation through a dominant negative TRAF-2 mutant potentiates the cytotoxic activity of TNF- α , suggesting that NF-kB is critical to the expression of protective protein(s) that inhibit TNF-induced cell death (**Liu et al, 1996**). Nf-kB activation by TRAF is mediated through phosphorylation of the I κ B, with subsequent degradation by the proteasome complex. NF-kB, released from the I κ B complex, enters the nucleus, activates various genes carrying the NF-kB response element and causes the onset of expression of apoptotic suppressive proteins (**Beg and Baltimore, 1996; Van Antwerp et al, 1996**). The situation, however, seems to be more complicated than initially expected. TRAF2 gene KO mice have been shown to display only a slight defect in their Nf-kB response to TNF- α (**Yeh et al, 1997**). Thus TRAF2 may not be essential for in vivo activation of Nf-kB. Alternatively there might be another member of the TRAF family that binds to TRADD and compensates for TRAF-2.

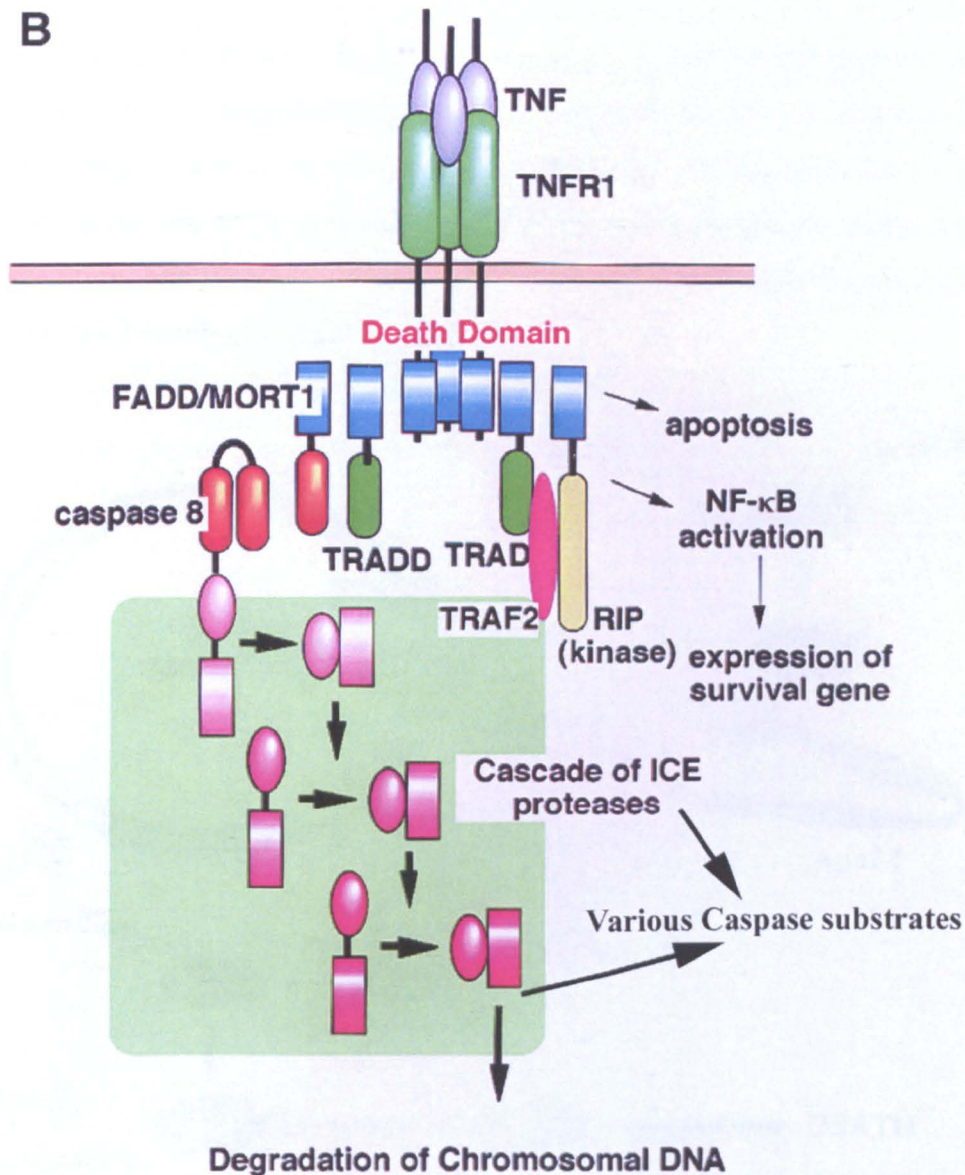


Figure 1.7) Model for TNF- α triggered apoptosis

TNF- α binds to TNFR1 and the trimerized receptor recruits TRADD via interactions between death domains. The death domain of TRADD then recruits FADD/MORT1 in a pathway leading to caspase8 activation, and subsequent ICE proteases cascade activation, which, as in the case for FAS, is able to activate the apoptotic program.

In parallel, another pathway causes the binding of RIP to TRADD transducing an apoptotic signal through their death domain. In addition RIP together with TRAF-2 activates the NF- κ B transcription factor, activating a protein synthesis dependent survival pathway.

Adapted from (Nagata, 1997)

In conclusion, the conservation of basal apoptotic machinery from the CED3-CED4-CED9 system in *C.elegans* to the highly complex system in mammals (involving the caspase family, the Apaf-1 system and the always increasing Bcl-2 family member) has been clearly shown. The growing Bcl-2 family can somehow appreciate multiple forms of intracellular damage and external contact stimuli and integrate these, sometimes competing signals to decide the fate of the cell. Other death signals, mainly those resulting from death receptor activation, find their way of access to the apoptotic machinery bypassing the steps controlled by Bcl-2 family members (Figure 1.8)

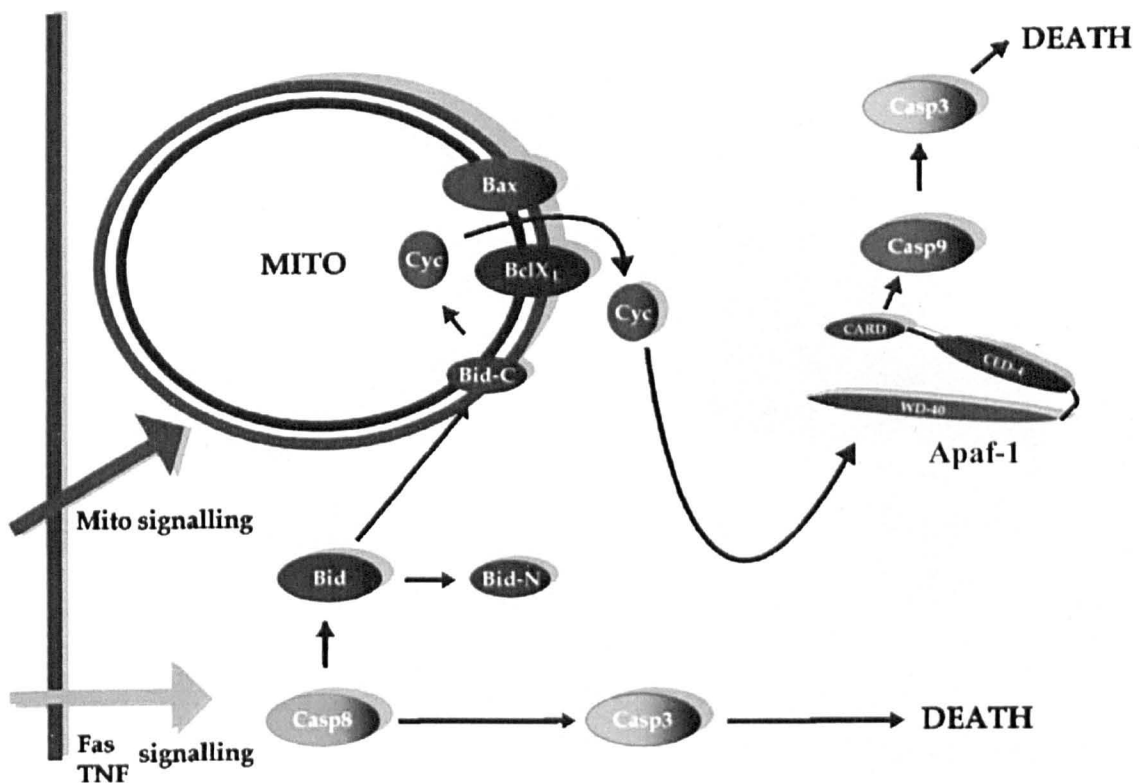


Figure 1.8) A summary of apoptosome regulation

Two pathways have been linked with apoptosome function: a Death Receptor-mediated signal that is mainly independent of Bcl-2 family members and that is able to directly activate the caspase cascade, and another one which is mainly based on a mitochondrial signalling, able to release Cytochrome c, activate Apaf-1 and thereby activating the caspase cascade. The two pathways are able to operate a cross-talk: caspase-8 activation by Fas and TNF- α has been reported to be able to cut the Bid protein and interact with the mitochondria based pathway, because a Bid fragment can induce the efflux of Cytochrome c into the cytosol. The balance between Bax, Bcl-x_L and other Bcl-2-like proteins regulates this efflux.

Adapted from (Cecconi, 1999)

The picture of the various interacting partners of TNFR1 grows in complexity, however the framework of the key events leading to cell death appears now quite well defined and is summarized in the **Figure 1.7**. An important and yet open field of discussion is still represented by the modulation of TNF- α apoptotic signalling by intracellular second messenger: of importance, in this context appears the role played by the activation of sphingomyelinases with ensuing generation of ceramide lipidic messenger and the role of nitric oxide in the regulation of the pathway.

Investigation of these regulatory pathways has been central to my thesis work: because of this I will provide a review of the knowledge about sphingomyelin cycle and of NO in the context of cell death, as presented in the next sections.

1.4) THE SPHINGOMYELIN CYCLE: AN INTRODUCTION

The notion of lipids being primarily inert structural components of cellular membranes resisted until the late 1970 s. Because of this, the discovery of the phosphoinositide cycle (**Berridge MJ, 1984**) represented a major breakthrough, pointing for the first time to lipids as key players in signal transduction events.

Phosphatidylinositol 4,5-bisphosphate was recognized to be hydrolyzed by phospholipase C (PLC) to release the hydrophilic head group inositol 1,4,5-triphosphate (IP₃) from the lipid backbone, 1,2-diacylglycerol (DAG). IP₃ interacts with an intracellular membrane receptor, the IP₃ receptor, leading to mobilization of Ca²⁺ from intracellular stores, whereas DAG directly binds to and activates protein kinase C (PKC) initiating a separate signal transduction cascade (**Nishizuka, 1995**). From that time onward other lipidic molecules found acceptance as second messengers and intracellular modulators: arachidonic acid (**Piomelli, 1993**), phospholipids (**An et al, 1998**), phosphatidic acid (**Daniel et al, 1999**) and phosphatidylinositol 3,4,5-tris-phosphate (**Chao and Olson, 1993**). More recently another class of lipids has been discovered as important signalling molecules: the sphingolipids.

Lipids of the sphingolipid class contain a long-chain sphingoid base backbone (such as sphingosine), an amide-linked, long-chain fatty acid and one of various polar head groups. The structure of these head groups defines the various classes of sphingolipid subtypes, with a hydroxyl group found in ceramide, phosphorylcholine in SM and carbohydrates in glycosphingolipids.

Richard Kolesnick and Yussuf Hannun's groups were the first to point attention to SM-based signalling pathways. The former group showed in 1987 the rapid activation of sphingomyelinases in response to 1,2 diacylglycerol, but not of phorbol ester treatment (**Kolesnick, 1987**) and proposed the existence of a SMase-based signalling pathway (**Kolesnick, 1989**); the latter group showed that this pathway could be activated by receptor-mediated mechanisms, and provided the first evidence for ceramide as a second messenger (**Okazaki et al, 1990**).

1.4.1) Enzymes in the sphingomyelin cycle

The sphingomyelin cycle is initiated upon activation of sphingomyelinases, specialized enzymes with phospholipase C activity that hydrolyze the phosphodiester bond of SM, causing the cleavage of a complex sphingolipid to ceramide and phosphorylcholine. The catabolic metabolism of ceramide proceeds through the action of ceramidase (CDases) with the formation of sphingosine, a sphingoid base that serves as substrate for sphingosine kinases to form sphingosine-1-phosphate, or that can be recycled into ceramide and complex sphingolipids through the action of ceramide synthases (**Figure** and **Hannun et al, 2001**). Ceramide can also be the starting point to generate back sphingomyelin. This occurs at the Golgi complex through the activity of sphingomyelin synthase, thus completing the sphingomyelin cycle, which, in conclusion, appears to be a very complex and regulated metabolic pathway.

In order to generate ceramide this de-novo synthesis pathway requires the coordinate action of serine-palmitoyl transferase and ceramide synthase. The reaction starts with a condensation reaction, which forms ketosphinganine from serine and palmitoyl-CoA: the rest of the pathway schematized in the following figure (**Figure 1.9**).

Alternatively, this pathway may re-utilize for ceramide synthesis the sphingosine released by sequential degradation of more complex sphingolipids. This pathway can be stimulated by chemotherapeutic drugs (daunorubicin, doxorubicin and many others) and ionizing radiations and appears to be activated hours after cell stimulation, resulting in a prolonged ceramide production. Because of this, it is unlikely to play a role in the early events of stress signalling (**Mathias et al, 1998**).

The SM pathway seems to be ubiquitous and evolutionary conserved. Most mammalian cells appear capable of signalling through the SM pathway. Receptors as diverse as those for CD28, CD95, TNF- α , IL-1 β , progesteron and glucocorticoid signal via the sphingomyelin pathway following activation by their respective ligands (**Spiegel and Kolesnick, 1996**).

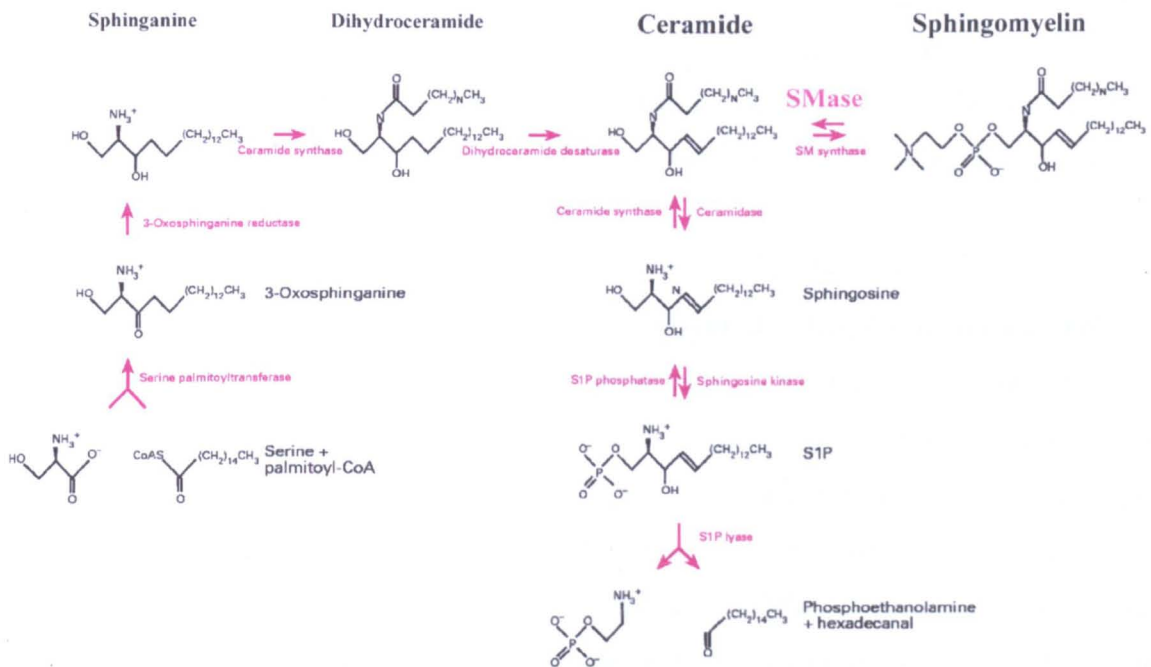


Figure 1.9) Sphingolipids structures and enzymes of metabolism

In terms of functional relevance, ceramide has been proposed to act as a coordinator of the response to various stresses inducing conditions (**Hannun, 1996**): this paradigm has been supported by the repeated finding that many inducers of stress response (not only those inducing apoptosis) result in transient or long-lasting ceramide accumulation, generated either by the activation of SMases (**Andrieu-Abade et al, 2001**) or the de-novo pathway (**Perry, 2001**), with possible involvement also of the ceramide clearing pathways (SM-synthase or CDases).

Attention will be mostly pointed to SMase since they are the central enzymes of SM cycle and are rapidly activated by stress stimuli (**Wiegmann et al, 1994; Cifone et al, 1995**). For this reason SMase are considered the principal players for the early production of ceramide in signalling events. Several isoforms of SMase have been reported, that can be distinguished mostly by their different optimum pH of activation. Some, but not all of these molecules have been identified at the molecular level. Among these particularly relevant appear Neutral SMase (N-SMase) and Acidic SMase (A-SMase) because they are rapidly activated by diverse stress stimuli, among which cytokines (TNF- α , Fas) and environmental stress (UV radiation, hypoxia). Moreover, their activity promotes an increase in cellular ceramide levels over a period ranging from minutes to hours, depending on the stimuli and on state of activation of the various enzyme of the cycle (**Hannun 1996; Hannun et al, 2001; Mathias et al, 1998**).

Human and murine Acid-SMase (pH optimum 4.5-5.0) have been cloned and determined to be the product of a single conserved gene: A-SMase was originally considered a strictly lysosomal enzyme because of its optimum pH at 4.5-5.0. However, another A-SMase isoform has been recently shown to localize to secretory vesicles near the plasma membrane (**Grassme et al, 2001**) and to be secreted extra-cellularly (**Schissel et al, 1998**). Other authors have reported the localisation of A-SMase in caveolae at the plasma membrane, which are micro-domains enriched in SM. In this compartment SMase can be activated by IL-1 β (**Liu and Anderson, 1995**). Relevance for cellular homeostasis of A-SMase comes once again from genetic studies: lack of a functional A-SMase gene has been shown to be the cause of a rare lipidic storage genetic dysfunction, the type A and B Niemann-Pick disease.

Niemann-Pick group of diseases has been infact subclassified in two categories, those with a primary deficiency in A-SMase activity (types A and B), and those with defective

intracellular processing and transport of low density lipoprotein (LDL)-derived cholesterol (type C): type A NPD patient suffers of progressive neurodegeneration during infancy, while nervous system involvement in type B is linked to the appearance of a cherry-red macula. In the two types of the A-SMase-linked NPD, the *in-vitro* assay for A-SMase have shown marked differences, with type-B derived cultured cells showing a significantly higher SM hydrolysis than type A. Moreover, also the brain lipid profile of Type A and Type B patients differs, with an increase of lysosphingomyelin, believed to be toxic to the nervous system, in type A patient versus type B patient (**Rodriguez-Lafrasse et al, 1999**). This property may account for the milder phenotype of Type B (for a more complete review of NPD diseases refer to **Kolodny, 2000**).

KO mice for A-SMase have confirmed its obligatory role for the phenotypes observed in the Niemann-Pick disease and, of importance, also for the dysfunctions at cellular level, both in the disease and its KO model. These dysfunctions include unresponsiveness, or limited response, to apoptosis triggered by a variety of stimuli, including UV radiation and TNF- α (**Lozano et al, 2001**).

N-SMases have their optimum pH of activation at 7.4 and are beginning now to be defined at the molecular level although the recent cloning by the Stoeffel's group has been questioned (**Hannun et al, 2001**; **Buccoliero and Futerman, in press**). Various different forms of neutral sphingomyelinases (pH optimum 7.5-8) have been reported, differing in their dependence on Mg²⁺ (**Levade and Jaffrezou, 1999**). What is clear, from the residual activity of A-SMase KO mice, is that the neutral enzyme(s) results from one or more distinct genes, other than that of A-SMase (**Horinouchi et al 1995**). Knowledge of the role of these enzymes is however still confused. In terms of activity it is important to note that N-SMase activity has been found to depend on the redox state of the cells and in particular on their levels of glutathione. The activity has been found to be de-repressed under conditions of oxidative stress, when glutathione is depleted, a condition that appears to happen after hours of stress inducing treatment, such as TNF- α treatment (**Liu et al, 1998**).

1.4.2) Ceramide and cell death

Evidence supporting ceramide as a messenger molecule in the context of cell death emerged from several independent studies (reviewed in **Mathias et al, 1998; Hannun et al, 2001**) and can be summarised as follows:

- i) In many cellular systems stress-induced increases in ceramide levels, classically measured by the diacylglycerol-kinase assay, precede biochemical and morphological features of apoptosis.
- ii) Increasing cellular ceramide levels by addition of natural ceramide, exogenous SMase or pharmacological agents that are able to interfere with the enzymes of ceramide metabolism are able to induce in the cell apoptotic features.
- iii) Genetic models, including Niemann-Pick disease cells, A-SMase knock out mice and glucosylceramide synthase knock out mice, display abnormalities in several aspects of cell death.

The observation that other sphingolipids molecules have not been reported to produce cytotoxic effects support the notion that ceramide has a central role in modulating and, in certain cases, in inducing some forms of stress-associated apoptosis. If the majority of the effects that have been described for ceramide imply its action as and be anti-proliferative and pro-apoptotic agent, an important class of molecules directly produced by ceramide catabolism, sphingosine-1-phosphate (S1P), has been implicated as a second messenger in cellular proliferation and survival (**Olivera and Spiegel, 1993**). Based on this information Spiegel and co-workers proposed the ceramide/sphingosine rheostat as a mechanism working to coordinately regulate proliferation and apoptosis through sphingolipids (**Cuvillier et al, 1996**). This model suggests that the fine regulation of the diverse enzymes of the SM cycle might be operational in the regulation of the various stress-induced apoptoses.

The recent discovery of a cell surface set of S1P receptors, the EDG proteins, adds a new molecular player in this signalling, suggesting that S1P might act not only intracellularly but also through a paracrine/autocrine action (**Hla et al, 2001**). This fascinating model and its operating mode await definitive confirmation.

Ceramide has been proposed to trigger the apoptotic pathway via two reported mechanisms: one involving the transcriptional activation of the JNK cascade and the other involving mitochondrial regulation.

JNK pathway, which is activated in response to several stress-inducing cytokines, UV, ionizing radiation and serum deprivation, lead to the phosphorylation of various transcription factors including c-Jun, with activation of transcription factor-2 (ATF-2) and CHOP/GADD 153 (**Fanger et al, 1997**). The role of ceramide in activating this pathway has been demonstrated in several studies. In fact, exposure of bovine endothelial aortic cells (BAEC) and monocytic cell line U937 to environmental stresses resulted in rapid ceramide generation, activation of JNK pathway and apoptosis (**Verheij et al, 1996**). Similarly, ceramide analogues stimulated JNK activation while disruption of the pathway with dominant-negative mutants of SEK-1 or c-Jun blocked TNF- α , stress- and ceramide-induced apoptosis. Ceramide generation may lead to JNK activation by at least two mechanisms: either via transforming-growth-factor β -activated kinase (TAK1) or via the small G-protein Rac-1. Shirakabe et al reported in fact that TAK1 is activated in response to ceramide or stress leading to ceramide generation, and that a kinase negative TAK1 blocked ceramide-induced JNK activation (**Shirakabe et al, 1997**). Gulbins and co-workers, on the other hand, showed that in Jurkat cells CD95 activation or ceramide treatment resulted in sequential activation of Ras, Rac-1 and the JNK pathway (**Brenner et al, 1997**), while ceramide and Fas have been indicated to activate phosphoinositide-3-kinase (Pi3-K) also in Jurkat and T cells (**Gulbins et al, 1998**). Thus, in response to stress stimuli, ceramide may utilize a variety of signalling relays to activate the JNK pathway (a pathway which is not only linked to cell death induction but also to effects unrelated to apoptosis) or even provide anti-apoptotic protection to the cells (**Liu et al, 1996**). This contradiction may be only apparent, being due to different actions of various JNK isoforms. In view of this, and until better reagents are available to distinguish between the various JNK isoforms, it is not possible to define unambiguously the role played by this ceramide-induced system in apoptosis induction.

Mitochondria have been shown to have a prominent role in the commitment phase of the apoptotic response and to represent a potential target for ceramide action. As described in the preceding section, mitochondria may function in the context of apoptotic cell demise through release of cyt-c, Apaf-1 and the generation of reactive oxygen species (ROS). Bcl-2 family members regulate signalling of the apoptotic responses through mitochondria in

many instances. Several reports show that Bcl-2 is able to inhibit ceramide-induced apoptosis, thereby suggesting a mitochondrial action of the lipidic messenger (**Farschon et al, 1997; Martin et al, 1995**).

Two distinct mitochondrial events have now known to be related to ceramide action: permeability transition and ROS generation. Even if it is a questioned point, induction of apoptosis has been frequently linked to the activation of MTPT, associated with the opening of large pores in the mitochondrial inner membrane with ensuing free diffusion of small molecules. In isolated mitochondria this phenomenon has been shown to be induced by a cytoplasmic fraction isolated from ceramide treated cells (**Pastorino et al, 1996**). From several studies this effect appears to be transcriptionally-independent (**Decaudin et al, 1997; Castedo et al, 1996**), stimulated by BAD and by signalled through a ceramide activated protein kinase (CAPK; **Basu et al, 1998**).

Also in isolated mitochondria ceramide has been shown to cause ROS formation, most likely through inhibition of the respiratory complex III. In fact inhibition of electron transport through complex II and I blocked, whereas inhibition of complex III potentiated the ceramide action. Further, TNF- α treatment was shown in one study to increase the level of ceramide in mitochondria, suggesting this event to be of physiological relevance (**Garcia-Ruiz et al, 1997**).

1.4.3) Death receptors and sphingomyelinases

Death receptors, in particular TNF-R55 of TNF- α , have been reported to induce activation of both A-SMase and N-SMase, with concomitant generation of bioactive ceramide. Most of the data were collected by studies with mutants of the receptor cytoplasmic domain, which suggested the existence of a link between receptor activation and SMase activation. The results are schematized in **Figure 1.10**.

A small motif of 11 aminoacids in the cytoplasmic portion of the TNF-R55 was found to be both necessary and sufficient for N-SMase activation (**Adam et al, 1996**). This motif, termed NSD, was shown to specifically bind a novel WD-repeat protein, FAN, that functionally couples TNF-R1 to N-SMase (**Adam-Klages et al, 1996**).

In contrast, the activation of A-SMase is due to a region at the C-terminus of the receptor corresponding to its death domain (**Wiegmann et al, 1994**), which serves as the binding domain for TRADD. The latter in turn is able to recruit other proteins, such as TRAF2, FADD and RIP. Over-expression of TRADD and FADD enhance TNF- α induced A-SMase activation, while neither TRAF2 nor RIP affects A-SMase activation (**Kolesnick and Kronke, 1998**).

Taken together, these observations suggest that the SMases are activated through distinct molecular mechanism and that this may affect distinct biological processes. With regard to programmed cell death, the activation of A-SMase through the death domain adaptor system indicates that A-SMase, rather than N-SMase, is involved in TNF-mediated apoptotic response. Deletion of the death domain region of TNF-R55 (**Wiegmann et al, 1994**) and CD95 (**Cifone et al, 1995**) blocked ligand-induced ceramide generation and the ensuing apoptosis

Evidence for a role of A-SMase in initiating the apoptotic response was provided also by genetic models of acidic SMase deficiency. Studies from the Kolesnick's group reported that lymphoblasts from patients with Niemann-Pick disease (NPD) and from acidic sphingomyelinase knock out mice show defects in apoptotic response (**Santana et al, 1996; Lozano et al, 2001**).

At the moment, the role of N-SMase is still much debated, however lack of appropriate reagents to investigate its role has made it difficult to interpret with certainty the results so far obtained.

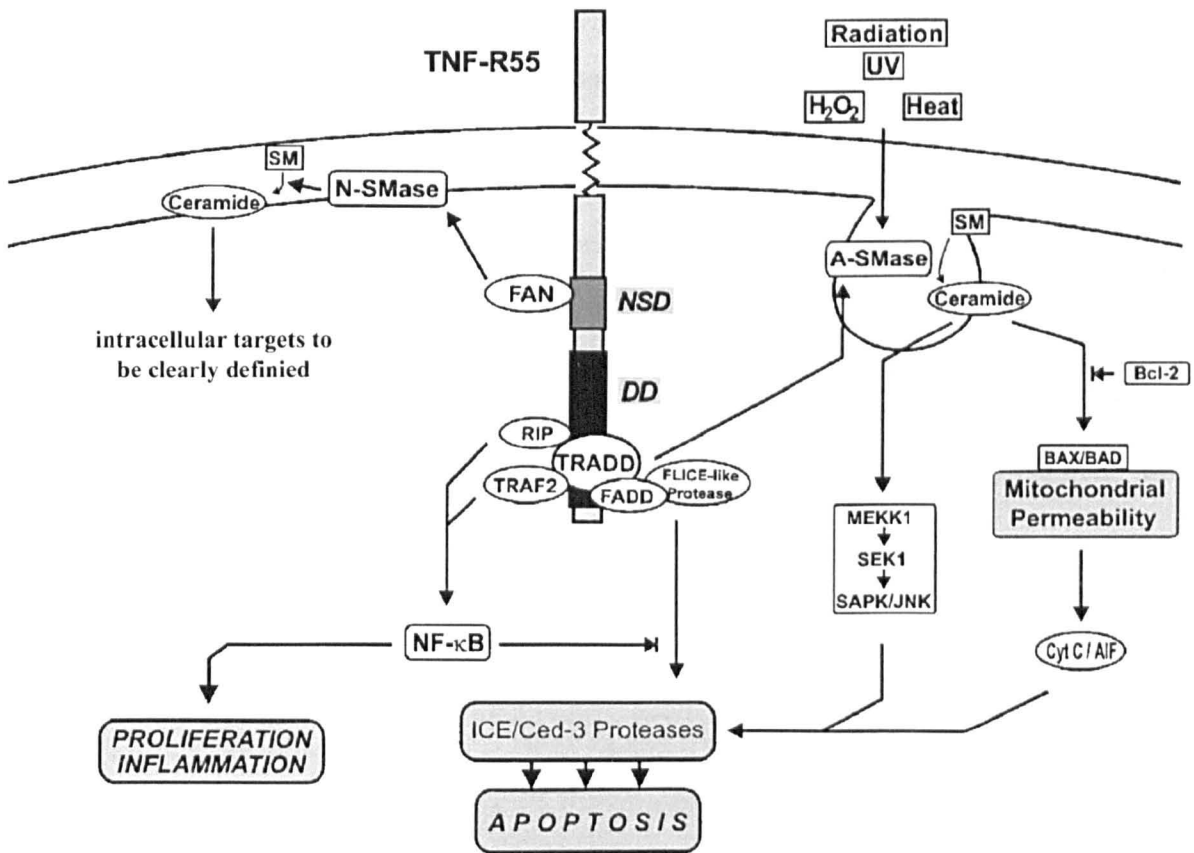


Figure 1.10) Proposed mechanism for TNF- α triggered activation of SMases

Different intracellular relays connecting TNF-R1 to different SMases isoforms: a membrane proximal region of the cytoplasmic domain links the adaptor protein FAN to N-SMase, while the assembly of a death domain adaptor protein complex links to A-SMase activation. Possible targets of A-SMase triggered ceramide formation have been depicted, with also some reported inhibitors of those effects, such as Bcl-2 on mitochondrial ceramide site of action. A-SMase has also been reported to be directly activated by environmental stresses, such as UV, X-Ray irradiation and heat.

Adapted from (Kolesnick and Kronke, 1998)

If the molecular intermediates between death receptor and SMase activation have been defined, at least in part, much of the transmembrane signalling system remains unknown: studies from our laboratory suggest that ceramide has a novel role in proximal events of cell death receptors activation (**DeNadai et al, 2000**). In particular, we produced evidence that exogenous ceramide is able to potentiate early TNF- α signalling, namely TRADD recruitment to TNF-R55 and caspase 8 activation, with ensuing enhanced apoptotic demise of the cells, evaluated by the DNA fragmentation index. This body of evidence will be described later on, after having reviewed the present knowledge about NO, in particular, the role of NO in the context of cell death. This because in the same work NO was been found to inhibit the effect of ceramide on early TNF- α signalling.

1.5) NITRIC OXIDE

The gaseous second messenger nitric oxide (NO) takes part in fundamental physiological processes, ranging from cardiovascular function to memory (**Dawson et al, 1992; Feldman et al, 1993; Ignarro, 1989; Moncada et al, 1991**) and to events correlated to microbicidal and tumoricidal responses (**Hibb, 1991; MacMicking et al, 1997**). An excessive and unregulated synthesis of NO, however, has been implicated as a causal or contributing factor in many pathological conditions, including vascular shock, stroke, neurodegeneration and chronic inflammation (**Gross and Wolin, 1995, Wink et al, 1998**). Due to its peculiar chemical nature, NO acts via a wide range of biological reactions, not restricted to binding to a few specific receptor molecules: it is a small, relatively stable, free radical gas able to readily diffuse into the cells and to react with its molecular targets. In this following sections I will introduce the generalities of NO, followed by role of NO in cell death, the specific subject of my thesis work.

1.5.1) Nitric Oxide: an historical perspective

NO has been known since the late 1970 s to be among the ligands able to activate soluble guanylate-cyclase (**Arnold et al, 1977**) and to cause vascular muscle relaxation via the synthesis of cGMP. The gas induces a transient, concentration-dependent relaxation of isolated strips of bovine coronary artery (**Gruetter et al, 1979**). In parallel, it was shown the existence of a factor produced by the endothelium, which had an obligatory role for the relaxation of arterial smooth muscle cells induced by acetylcholine administration (**Furchgott and Zawadzki 1980**). This factor was named the endothelium derived relaxation factor (EDRF).

The classical works by Furchgott, Ignarro (reviewed in **Furchgott , 1988**) and Moncada (**Palmer et al, 1987**) lead to the identification of NO as ERDF, and triggered an avalanche of independent studies carried out in many diverse directions by hundreds of different investigators, that unraveled the role of this small molecule in the context of various biological processes, starting from the observation that tumour cells can be killed by an L-arginine dependent activity exerted in activated rodent macrophages (**Hibbs et al, 1987**) and that the cytotoxic agent responsible for this activity was indeed NO (**Stuehr and Nathan, 1989**).

Another area in which a physiological role for NO was independently discovered was neuronal signalling in response to NMDA receptor activation: Garthwaite and co-workers established that cGMP levels increase in cerebellar granule cells in response to glutamate binding to NMDA receptor, and demonstrated the release of a guanylyl cyclase-activating factor with biological activity and stability characteristics similar to those of NO/ERDF (Garthwaite et al, 1988).

1.5.2) Nitric Oxide Synthases: isolation and classification

Following the characterisation by the Moncada's group of the enzymatic pathway leading to generation of NO from L-arginine (Palmer et al, 1988), a big effort was addressed to the identification of the enzyme molecules responsible for the synthesis of NO. Bredt and Snyder were the first to purify from the rat cerebellum an enzyme able to synthesize NO from the stoichiometric conversion of L-arginine to citrulline in the presence of Ca^{2+} and calmodulin (Bredt and Snyder, 1990). Thereafter, Bredt and co-workers cloned the enzyme (Bredt et al, 1991) showing that its C-terminal shows a homology (40% aminoacidic identity; 60% homology) to NADPH cytochrome P450 reductase, and that consensus sequence for NADPH, FAD and FMN binding sites are present within this enzyme, called neuronal Nitric Oxide Synthase (nNOS).

The two other known isoforms of NOS were then identified based on degenerate nucleotide probes from bovine aortic cells (Sessa et al, 1992) and from mouse macrophages (Xie et al, 1992).

Nomenclature of these enzymes has been a matter of debate. The most widely accepted identifies the three enzymes from the tissue employed first for cDNA isolation and cloning:

- i) nNOS was originally purified and cloned from the CNS, but now is known to have a more wide distribution, with an important level of expression in skeletal muscle. This isoform is also named NOS I
- ii) inducible-NOS (iNOS) was originally purified and cloned from an immunoactivated macrophage cell line. Since then it has been identified in mammals cells as diverse as cardiac myocytes, glial cells and vascular smooth muscle cells. This enzyme is also named NOS II.

- iii) Endothelial-NOS (eNOS, also named NOS III), the last of three mammalian isoforms to be identified, was originally purified and cloned from vascular endothelium. Since then it was discovered in various other cell types: e.g. cardiac myocytes, blood platelets, hippocampal neurons and T-lymphocytes.

Although the sequences of the various NOS isoforms are highly conserved across species (from 80 to 94% identity), between any two isoforms identity is only 50-60% (**Sessa 1994**). Remarkably, the regions of high homology are associated with the cofactor binding sites, strongly pointing to a conservation of the structure-function relationship in this area in the codified proteins. The NOS proteins contain also a haem and its putative binding site is localized to the N-terminal region (**Klatt et al, 1992**). nNOS is characterised by the presence of a N-terminal region longer than that of the other isoforms, containing protein-protein interaction domains (such as PDZ domains) which are crucial for its binding to other proteins (see next section)

The sequence homology between the C-terminal half of NOS and cytochrome P450 reductase, and the presumptive requirement of a haem binding site in the N-terminal portion of NOS suggested that NOS is composed of a distinct reductase (C-terminal) and an oxygenase (N-terminal) domains. This feature was found to be crucial for the catalytic properties of the enzymes (**Figure 1.11**)

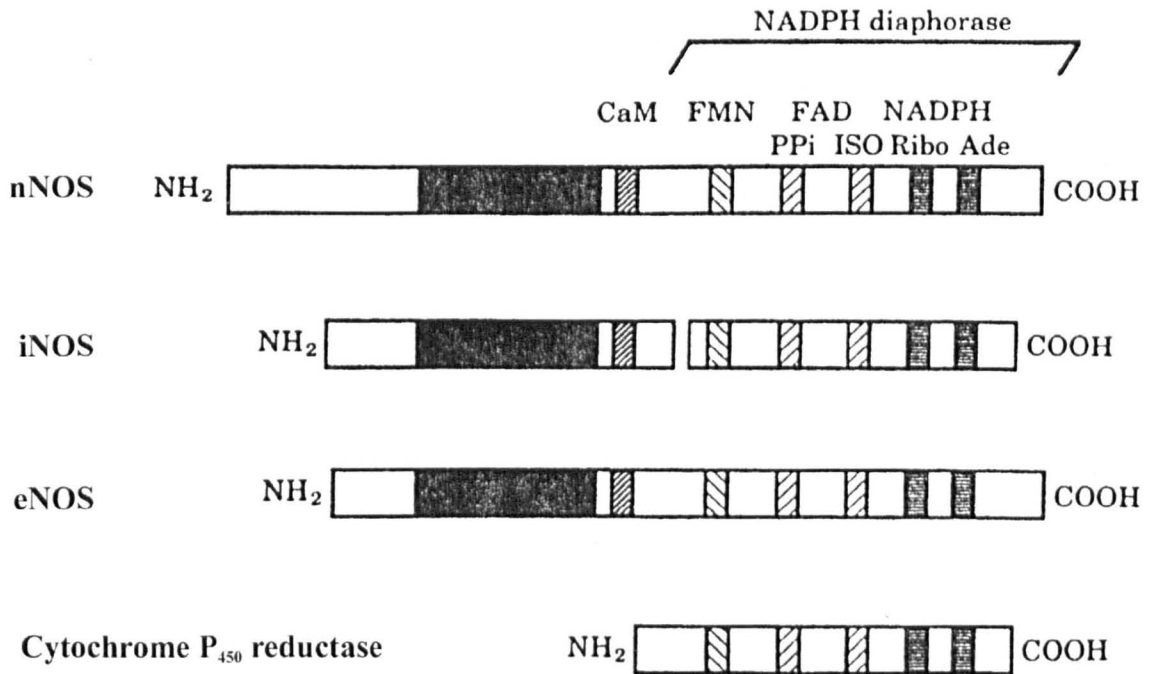


Figure 1.11) Relationship among the polypeptide sequence for NOS isoforms and cytochrome P450 reductase

The consensus sequences for the binding of the various cofactors to the NOS isoforms are labelled: NADPH (Ade: adenine; Ribo: ribose); FAD (ISO: isoalloxazine; Ppi: pyrophosphate); FMN and CaM (calmodulin). The darkened N-terminal region shows 65-71% sequence identity between the three isoforms and contains the putative L-arginine-binding region and the binding site for tetrahydrobiopterin and haeme. The gap in iNOS sequence represents a 40 aminoacid deletion.

Adapted from (Griffith and St her, 1995)

1.5.3) NOS: catalytic mechanism

Generation of NO by the three different NOS occurs via a common, similar catalytic scheme, involving the five electron oxidation of the terminal guanido nitrogen of L-arginine to form NO plus L-citrulline. Such reaction is complex, involving molecular oxygen and NADPH as co-substrates, with the concomitant presence of numerous other redox cofactors including the enzyme-bound haem, reduced thiols, FAD, FMN and tetrahydrobiopterin.

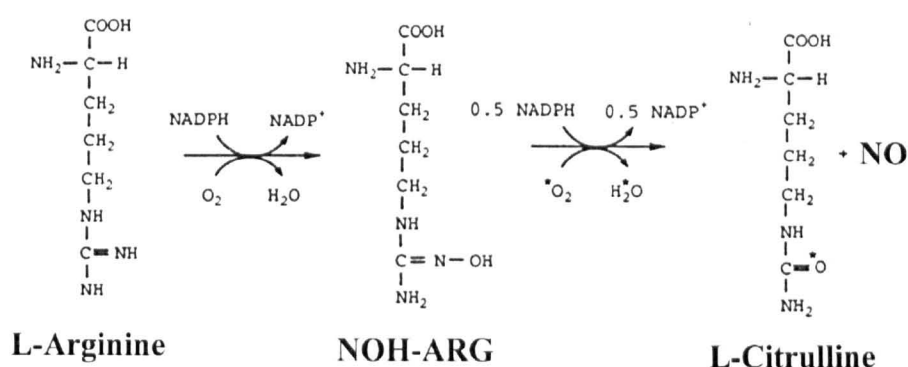


Figure 1.12) Schematic representation of NOS reaction sequence

The reaction sequence shown in the above equation (adapted from **Griffith and St ehr, 1995**) represents two successive mono-oxygenase reactions, both being mixed functions oxidations in which the four electrons necessary to reduce O_2 are derived from both NADPH and the aminoacid substrate. In the first reaction, two electrons are contributed by NADPH, and a guanidino nitrogen of arginine undergoes a two electron oxidation, with formation of the enzyme bound NOH-arginine (NOH-ARG) intermediate. In the second, one electron is contributed by NADPH, and NOH-arginine undergoes a three electron oxidation to finally form citrulline and NO. The details of nitric oxide synthase activity are not yet completely understood but represent still a matter of debate for biochemist (for complete review see **St ehr, 1999**).

In order for this complex reaction to proceed, the reductase and the oxygenase domains of NOS have to be positioned in a well-defined, three-dimensional organisation, with

calmodulin being a critical player in the process. The consensus sequence for calmodulin binding is near the center of NOS, separating the above-mentioned domains: Abu-Soud and St uher argued that the calmodulin binding domain acts a bridge between the reductase and the oxygenase domain, and that the binding of calmodulin represents a sort of switch for the activity of the enzymes (**Abu-Soud and St ehr, 1993**). When Ca^{2+} /calmodulin is not bound to the protein, the domains are not aligned and the reductase domain cannot supply electrons to haem. Instead, when Ca^{2+} /calmodulin is bound, the domains align and the enzyme is fully active (**Figure 13**)

For all three NOS isoforms, NO synthesis depends upon the enzyme binding of calmodulin. For eNOS and nNOS increases of intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) are required for calmodulin binding and, consequently, for enzyme full activation. By contrast iNOS appears able to bind calmodulin, and hence become fully activated, even at the low $[\text{Ca}^{2+}]_i$ characteristic of the resting cells. This feature gives rise to a profound biochemical difference between the three different isoforms, also reflected by their functional biological properties. Intracellular activity of eNOS and nNOS is precisely and closely regulated by transient changes in $[\text{Ca}^{2+}]_i$, whereas the activity of iNOS in immunoactivated cells is dependent on its onset of expression and does not depend on $[\text{Ca}^{2+}]_i$ transients (**Nathan and Xie, 1994**).

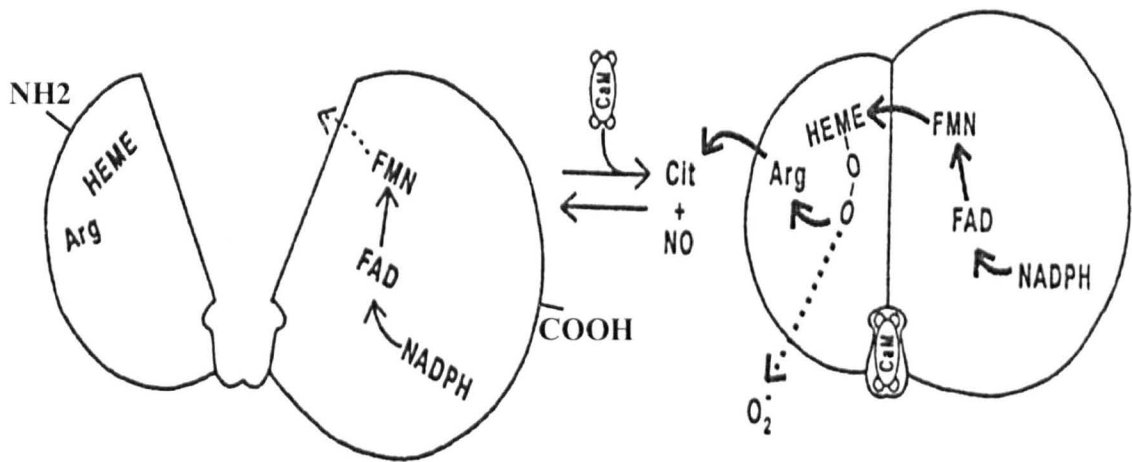


Figure 1.13 Central role of calmodulin (CaM) in NOS activity

This schematic model highlights the key role of CaM in aligning the oxygenase (N-terminal) and reductase (C-terminal) domains of NOS. The black arrows indicate the direction of the electron flow. CaM with four associated Ca^{2+} atoms binds to a region in the middle of the two domains and, so doing, it is able to align them and allow electron transfer from FMN to haem. In the absence of arginine, molecular oxygen ($\text{O}-\text{O}$) can dissociate from haem as superoxide (O_2^-)

Adapted from (Griffith and St ehr, 1995)

1.5.4) NOS: sub-cellular localization and function

Another important feature of NOS enzymes is represented by their subcellular localization, since localization within the cell might be expected to influence the biological role and chemical fate of produced NO.

Of the three isoforms, iNOS and nNOS have been reported to be soluble (cytosolic), while eNOS have been found to be primarily localized at particulate sub-cellular fractions, such as plasma membrane and Golgi fractions (**Hecker et al, 1994; Sessa et al, 1995**).

This distinction is not so clear-cut since important protein-protein interactions have been reported also for nNOS and iNOS that may have an important role also in their localization. The N-terminal domain of nNOS contains a PDZ domain, a protein-protein interaction sequence, which scaffolds the enzyme to integral membrane and cytoskeletal proteins of skeletal muscle, such as the dystrophin complex (**Brenman et al, 1995**) and α -1 syntrophin, and neurones (PSD95, see **Brenman et al, 1996**). Another protein target for PDZ domain interactions with nNOS has been identified as the muscle isoform of phosphofructokinase (PFK-M) in skeletal muscle and neurons (**Firestein & Bredt, 1999**). The functional importance of these interactions is still under investigation.

Using an iNOS-GAL-4 DNA binding domain fusion as a bait to screen a GAL4 activation domain-tagged, mouse kidney cDNA library by the two-hybrid method, the group of Kone has identified an interaction between iNOS and the Rho family GTPase Rac2 (**Kone and Kuncewicz, 1998**). Functional studies have indicated that such interaction may be important in regulating iNOS activity. The authors propose that Rac2 serves as an allosteric activator of iNOS in macrophages.

The most complete body of information is referred to eNOS, originally described as a protein localized in discrete subcellular localization. eNOS does not contain any hydrophobic transmembrane domain, and its plasma membrane localization is mediated by acylation. In fact, eNOS is co-translationally and irreversibly myristoylated at an N-terminal glycine residue while palmitoylation occurs post-translationally, and reversibly, at cysteine residue Cys¹⁵ and Cys²⁶. These modifications have been clearly demonstrated to be crucial for eNOS targeting to plasma membrane and to Golgi region. More precisely, myristoylation targets the protein to the Golgi apparatus where it is palmitoylated. The double acylated protein is then targeted to the plasma membrane, where it colocalizes with the resident integral membrane protein of caveolae, caveolin-1 (**Shaul, 2002**).

Localization of eNOS appears to be an important feature for enzyme regulation. In the plasma membrane rafts, caveolin-1 has been reported to interact with eNOS by a direct protein-protein interaction (**Li et al, 1996**), thus inhibiting the eNOS activity both *in vitro* and in endothelial cells (**Garcia-Cardena et al, 1997; Michel et al, 1997**).

The interaction of calmodulin and/or caveolin-1 with eNOS has been shown to be mutually exclusive, suggesting a dynamic regulation of the enzyme by $[Ca^{2+}]_i$. At rest eNOS is tethered to caveolin-1 and inactive. After $[Ca^{2+}]_i$ increase, calmodulin binds to and caveolin dissociates from eNOS resulting in an activated eNOS/calmodulin complex. When the $[Ca^{2+}]_i$ wave subsides, the cycle is reversed, with calmodulin dissociating and caveolin re-associating with the now inactive eNOS (**Michel et al 1997**). This regulation of activity by caveolin-1 may have implications relevant also to pathological situations: for example, serum from hypercholesterolaemic patients increases the expression of caveolin-1 and the formation of caveolin-eNOS heterocomplex in endothelial cells, thereby limiting both the basal and the agonist stimulated NO release (**Feron et al, 1999**). This may represent a rationale for the endothelial dysfunction in hypercholesterolaemia.

1.5.5) NOS: modulation of activity through phosphorylation

Another important post-translational modification of eNOS is phosphorylation, which has been found to regulate enzyme activity. This is of particular interest as it would potentially represent a means to correlate NOS activity with other signalling pathways other than the classical $[Ca^{2+}]_i$ rise dependent mechanism.

Even in this case eNOS represent the most tightly regulated enzyme. In fact it has been clearly shown that fluid shear stress elicits phosphorylation and activation of eNOS (**Corson et al, 1996**). Further studies have shown that eNOS phosphorylation occurs on Ser1¹¹⁷⁹, by serine/threonine AKT kinase (**Dimmeler et al, 1999; Fulton et al, 1999**). The mechanism underlying this Ca^{2+} -independent activation of the enzyme is still unknown.

The proposed mechanism is that the Akt mediates phosphorylation results in an increased electron flux through the reductase domain of the enzyme, and thus in increased NO production (**McCabe et al, 2000**). This activation seems to be Ca^{2+} -independent. This definition, however, might be misleading, in the sense that the Ca^{2+} /CaM complex is always necessary to induce NO production. A possible explanation is that phosphorylation acts by lowering the level of Ca^{2+} required to activate the enzyme.

Other than shear stress, a variety of stimuli as diverse as estrogen (**Haynes et al, 2000**), vascular endothelial growth factor (VEGF, **Fleming et al, 2001**), insulin (**Montagnani et al, 2001**) and sphingosine-1-phosphate (**Igarashi et al, 2001**) have been linked to eNOS activation through activation of Pi3K/AKT. Other phosphorylation sites and protein interactions have been proposed to be important. In these cases, however, the framework is still quite confused, and further investigation is necessary to have the final picture (see **Fulton 2001** for a review).

Modulation through phosphorylation has been also indicated for nNOS: in this case the Ser847 phosphorylation operated by CAM Kinase II has been linked to a decrease in NOS activity (**Hayashi et al, 1999**). No report to date, instead, has linked iNOS regulation to phosphorylation of relevant sites.

1.6) NITRIC OXIDE AND CELL DEATH

NO is an important regulator of cell death, however with intriguing properties. The gas, in fact, has been shown to have both detrimental effects on cell viability, as well as protective effects in various paradigms of cell death. This bi-univoque behaviour was mainly correlated to the source and with the mode of NO production: NOS isoenzyme specific activity allowed to discriminate between a low and regulated versus a high and massive output of NO production, and to establish a rough correspondence between homeostatic and toxic functions of the gaseous messenger.

1.6.1) NO as a pro-apoptotic agent

The first reports of detrimental effects, such as lysis of islet cells by activated macrophages through an arginine dependent NO generation, came in the early nineties (**Kroncke et al, 1991**). Indications of NO-mediated apoptosis were independently reported by several groups in 1993 (**Albina et al, 1993**; **Sarih et al, 1993**; **Xie et al, 1993**); evidence for NO-induced apoptosis in macrophages was provided by chromatin condensation and inter-nucleosomal DNA fragmentation, and its involvement was confirmed by preventing these features by an L-arginine restricted medium or by the presence of the NOS inhibitor, L-NMMA.

In various cells the production of high levels of NO is associated with cytotoxicity: iNOS-generated NO stimulates apoptosis in ascite hepatoma cells in tumor bearing rats (**Nishikawa et al, 1998**) and NO causes autolysis in tumor cells and bystander cells (**Xie et al, 1997**). Furthermore, macrophages from septic shock mice exhibit increased apoptosis that was rescued by NOS inhibition (**Williams et al, 1997**). A functional role of stimulated NO production was also established in T-Cell Receptor (TCR)-triggered apoptotic death of mature T-lymphocytes (**Williams et al, 1997**).

Following these initial observations, an avalanche of reports have confirmed the ability of NO to induce cell death in cellular models, as diverse as B-cells, thymocytes, chondrocytes, neurons, vascular endothelial cells and other. In all of these cases the detrimental effects were related to the iNOS-induced expression and massive chemical production of NO. High concentrations of NO were correlated to p53 (the principal cell cycle controller and

apoptosis initiator) accumulated at an early time point, preceeding DNA fragmentation. Such a p53 accumulation was attenuated by blocking NO production (**Brockhaus and Br ne, 1999**). There are indications that the proposed accumulation of p53 by NO is sustained by activation of the MAPK signalling pathway (**Callsen and Br ne, 1996**), but the exact causal relationships are yet to be clarified.

Another important player in NO-induced cytotoxicity is represented by Bcl-2 family members: in several studies, NO-evoked apoptosis and decreased expression of anti-apoptotic Bcl-2 family members were correlated, while expression of the pro-apoptotic protein Bax was increased during NO-induced apoptosis. Macrophages transfected with human Bcl-2 and showed protection against various NO donors and endogenously generated NO, although in these cases p53 accumulation remained unaltered. These results show that Bcl-2 acts downstream of p53 and upstream of cyt-c release, that was instead attenuated (**Messmer et al, 1996; Bonfoco et al, 1997; Melkova et al, 1997**).

A key player in apoptosis induction, as already mentioned, is represented by mitochondria. Several indications exist in fact of an effect of NO on mitochondrial function, with a fall-out consequence on cell death. The NO-dependent, macrophage-induced cytotoxicity of tumor cells has been associated with characteristic sets of metabolic changes in various cell types, in particular inhibition of mitochondrial aconitase, complex I, complex II and nuclear DNA synthesis. These changes are reproduced by incubating the cells anaerobically with NO, suggesting an effect of NO on the iron sulphur centres of mitochondrial enzymes. It is not yet completely known whether these effects are ascribed directly to NO, or to its derivative peroxynitrite (ONOO.-) (for a complete review, see **Brown, 1999**).

Treatment of macrophages with NO donors causes a reversible inhibition of respiration, while treatment for several hours induces an irreversible blockade which appears to be due to the inhibition of complex I (**Clementi et al, 1998**). Remarkably, induction of iNOS caused an irreversible inhibition of mitochondrial respiration in a variety of cell types: in fibroblasts, interferon- γ -dependent induction of iNOS expression caused inhibition of respiration by an arginine dependent process (**Dijkmans et al, 1991**); in cultured astrocytes or microglia, the same expression caused an irreversible inhibition of complex I, which could be prevented by NOS inhibitors (**Bolanos et al, 1994**).

The NO-dependent cell damage might be ascribed to various effects of the gas: inhibition of respiration, depolarisation of mitochondrial membranes, release to the cytosol of mitochondrial apoptogenic factors (such as cyt-c) in the cytosol, cell oxidation and

activation of the apoptotic machinery (**Hortelano et al, 1997**). The mechanism of the NO-induced apoptosis appears, however, to depend upon many other conditions accompanying, but not due to, NO. ATP levels are crucial for a switch between apoptosis and necrosis, and NO has been shown to induce apoptosis or necrosis depending on the [ATP] levels (**Richter et al, 1997**). The presence of glucose and the rate of glycolysis of each single cell can also be crucial to the NO responses, as cells can survive the inhibition of mitochondrial respiration caused by induction of iNOS as long as glucose is present and ATP levels are maintained (**Geng et al, 1992**).

Also the inhibition by NO of cytc-oxidase might play a role in the cytotoxicity of iNOS-expressing cells toward bacteria, tumour cells, host cells and iNOS expressing cells themselves. iNOS expressing cells in culture can in fact reversibly inhibit various cellular respiration by this means. The reversible inhibition might be then turned to an irreversible and cytotoxic inhibition by a variety of factors, which might include necrosis caused by ATP depletion, opening of the permeability transition pore, and Ca^{2+} release from the mitochondria.

Pathological situations, mimicked for example by stimuli such as LPS, a potent immunostimulator, induce expression of a wide array of genes, as well the release of inflammatory cytokines and NO. All these effects have been linked to apoptosis in macrophage cell lines and in primary lines (**Lakics and Vogel, 1998**).

Indeed, in conclusion, several observations indicate that iNOS induction and apoptosis are positively correlated. Moreover, positive correlations exist between NO and important pathological conditions such as the development of atherosclerotic lesions (**Kinscherf et al, 1999**), osteoarthritis (**Borderie et al, 1999**) and lupus nephritis (**Wang et al, 1997**), pointing to an importance of iNOS expression and NO synthesis in relevant human diseases.

Despite the intensive work, the exact mechanism(s) by which NO kills cells is(are) still not perfectly clear (see **Figure 1.14** for a schematic summary of the current knowledge); moreover a large literature shows that NO is also cytoprotective as reviewed in the next section.

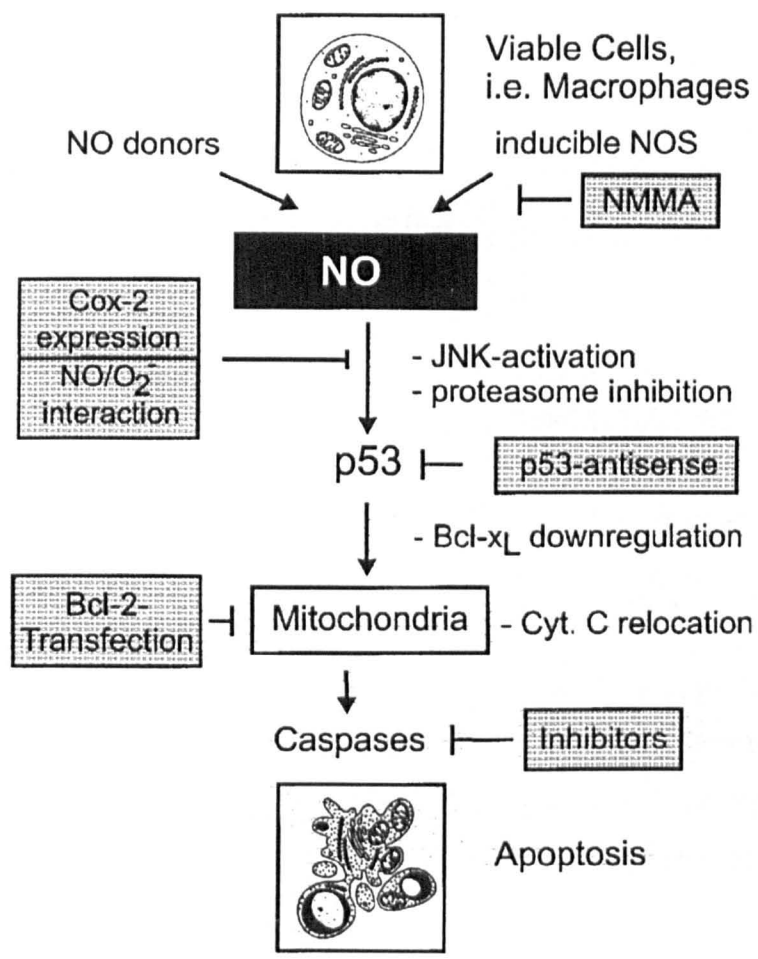


Figure 1.14) Signaling components characterizing NO-triggered apoptosis

In this summary scheme there are indicated several signalling components on NO mediated cell death, as well as some possible antagonistic interventions (grey boxes). It is important to note that the arrows don t imply a direct cause-effect relationship between the indicated components.

Adapted from (Br ne et al, 1999)

1.6.2) NO as an anti-apoptotic agent

The first demonstration of protective effects exerted by NO was obtained first in B-lymphocytes (**Mannick et al, 1994**) and later shown in a variety of other cell types (reviewed in **Liu and Stamler, 1999**). Also iNOS-generated NO may be protective. In fact, both Epstein-Barr virus (EBV) infected B cells, as well as EBV negative Burkitt's lymphoma cell lines, express iNOS constitutively and the inhibition of the basal enzyme activity with L-NMMA or by substrate depletion causes an increase in the rate of spontaneous apoptosis. This increase of apoptosis was partially blocked by administration of L-, but not D-, arginine.

NOS not only protect B cells against spontaneous apoptosis but also from apoptosis induced by Fas activation (**Mannick et al, 1997**); the same is true also for T cells and monocytes (**Sciorati et al, 1997; Mannick et al, 1997**). Protective effects of NO were also demonstrated in hepatocytes, where L-NMMA and L-NAME, but not the iNOS inhibitor L-N⁶-(1-iminoethyl)lysine (L-NIL), potentiated LPS-induced necrosis (**Ou et al, 1997**). In addition, the NO donor S-nitroso-N-acetyl-penicillamine (SNAP) suppressed both spontaneous hepatocyte apoptosis and that induced by TNF- α or anti-Fas antibody in vitro (**Kim et al, 1997; Saavedra et al, 1997**). NO donors have also been reported to inhibit apoptosis in both human neuroblastoma and rat PC12 cell lines (**Farinelli et al, 1996; Lievremonet et al, 1999; Ogura et al, 1998**).

Cytoprotection by NO appears of physiological relevance also in the endothelium: it has been shown that shear stress, an important trigger of eNOS activity *in vivo*, protects human umbilical venous endothelial cells from apoptosis induced by TNF- α (**Dimmeler et al, 1997**), oxidative stress (**Hermann et al, 1997**) or oxidized LDL (**Dimmeler et al, 1999**).

The protective role of eNOS-generated NO has been clearly shown also in rat embryonic motor neurons, where only eNOS is constitutively expressed (**Estevez et al, 1998**). Application of L-NAME had no effect but resulted in apoptosis when motor neurons were cultured in the presence of brain-derived neurotrophic factor (BDNF).

Multiple mechanisms, either dependent or independent on cGMP production, have been proposed to explain the protective role of NO in the above-mentioned cell systems. Among the cGMP independent protective effect, particularly relevant appears the inhibitory S-nitrosylation of the cysteine catalytic site of caspases, as shown for different classes of NO donors as well as for endogenously generated NO. For example, SNAP efficiently S-

nitrosylates recombinant caspase-3 (**Kim et al, 1997**), caspase 1 and caspase 8 (**Dimmeler et al, 1998**).

Nitrosylation is however a very questioned issue in the field, and there is debate as to whether the process is real and widespread *in vivo*, since it seems to require particular conditions that seldom occur under physiological conditions. An important prerequisite for nitrosylation to occur is that the cell reducing potential (maintained mainly by GSH) is low. In the case of the TNF- α triggered death this appears to occur only much later than the application of apoptotic stimuli (**Higuchi et al, 1998**).

Another possible protection mechanism by direct action of NO has been suggested to be the consequence of reversible inhibition of mitochondrial respiration exerted by nanomolar concentration of the gas. In particular, inhibition of respiration at complex IV leads to a sort of temporary freezing of the mitochondrial apoptotic machinery, as it stimulates reversal of mitochondrial ATPase, with maintenance of the mitochondrial membrane potential (**Beltran, et al, 2000**).

cGMP-dependent effects have been clearly shown in some cellular systems. In rat motor neurons eNOS protection was shown to be dependent on soluble guanylate cyclase: ODQ, an inhibitor of soluble guanylate cyclase, induced apoptosis in cells treated with BDNF. Such an effect was relieved only by treatment with cGMP analogues (**Estevez et al, 1998**). Similar observation has been made in PC12 cells (**Farinelli et al, 1996**), hepatocytes (**Kim et al, 1997**) and $\gamma\delta$ -T lymphocytes (**Sciorati et al, 1999**). The effect of the NO-triggered cGMP accumulation may be mediated through cGMP-dependent protein kinase (**Kim et al, 1997**). The precise mechanism of action of this enzyme in apoptosis prevention is however still not known. Possible target of the NO/cGMP protective action have been identified at the level of the mitochondria: inhibition of cyt-c release (**Li et al, 2000**) and collapse of the mitochondrial membrane potential (**Takuma et al, 2001**).

Moreover, a cGMP dependent protection could be due to the suppressed expression of the protein BNIP3 (**Zamora et al, 2001**), a pro-apoptotic protein of the BH3 domain-containing BCL-2 family (including BIK, BID and BAD), localized to mitochondria and other cytoplasmic membrane structures (**Boyd et al, 1994**). The exact mechanism of BNIP3-mediated cell damage still remains unknown, but might be due to formation of heterodimers with anti-apoptotic proteins (**Yasuda et al, 1998**). Since BNIP-3 is a pro-apoptotic protein, suppression of its expression by NO/cGMP may contribute to the anti-apoptotic activity of NO.

Evidence produced in our lab has shown that in $\gamma\delta$ -lymphocytes, the sensitivity to CD95-induced cell death was progressively acquired, and that this feature was directly correlated with the downregulation of eNOS. T cells treated after 14 days of propagation (when eNOS is still present) were significantly resistant to CD95 activation. This resistance was relieved by the treatment of the cells with haemoglobin (a nitric oxide scavenger), with L-N-(iminoethyl)-ornithine (L-NIO), a NOS inhibitor, and by pretreatment with the soluble guanylate cyclase inhibitor, ODQ. Of importance, in these cells NO/cGMP was able to inhibit the first wave of ceramide production, indicating the importance of NO in the control of the levels of this pro-apoptotic lipidic messenger. After 21 and 28 days of restimulation, $\gamma\delta$ -cells lost eNOS expression and acquired sensitivity to CD95 treatment (Sciorati et al, 1999).

Taken together these results indicate that NO/cGMP generated from endogenous eNOS is able to protect from CD95-triggered apoptosis, possibly through a ceramide dependent step. The intracellular pathways of this cGMP-dependent action of NO, and its ultimate target, remain to be established.

1.7) AIMS OF THE THESIS

A recent paper by our group identified a previously undescribed site of possible regulation of apoptotic signalling by NO/cGMP (**DeNadai et al, 2000**). In the case of apoptosis triggered by TNF- α , cGMP-dependent protection by NO involves inhibition of proximal events of the apoptotic cascade triggered by the cytokine, i.e. recruitment to TNFR1 of the adaptor protein TRADD (**Figure 1.15**), and ensuing activation of the initiator caspase 8. We found that this effect of NO/cGMP was due to the impairment of the TNF- α -triggered generation of pro-apoptotic lipid messenger ceramide, occurring in the first minutes after cytokine treatment of the cells (**Figure 1.16**). We also found that this inhibition correlated with the NO/cGMP triggered modulation of DNA fragmentation triggered by the treatment with TNF- α . (**Figure 1.17**)

This body of data represented an important novel finding, since regulation by intracellular messengers of receptor events, never reported previously, might represent a very important keypoint of regulation of apoptosis, at a point where even small changes might result in substantial downstream consequences.

- The first aim of the present thesis was to evaluate which might be the target of NO/cGMP in the regulation of these proximal events in U937 cells. To answer this question we have studied the effect of NO administered via exogenous chemical donor.
- The other aim of the thesis was to establish and characterize a model to study directly the role endogenously produced NO in the modulation of TNF- α -triggered apoptosis. To this aim we have engineered a HeLa tet off cell line, in which we could control eNOS expression by controlling the concentration of doxycycline in the culture medium. This cellular model is particularly useful to directly test the effects of regulated expression of eNOS on several biological endpoints in a system that failed to express endogenous NOS and to evaluate the direct role of expression of a the enzyme.

Results obtained have been subdivided in two sections describing experiments carried out to fulfil each of the two specific aims outlined above.

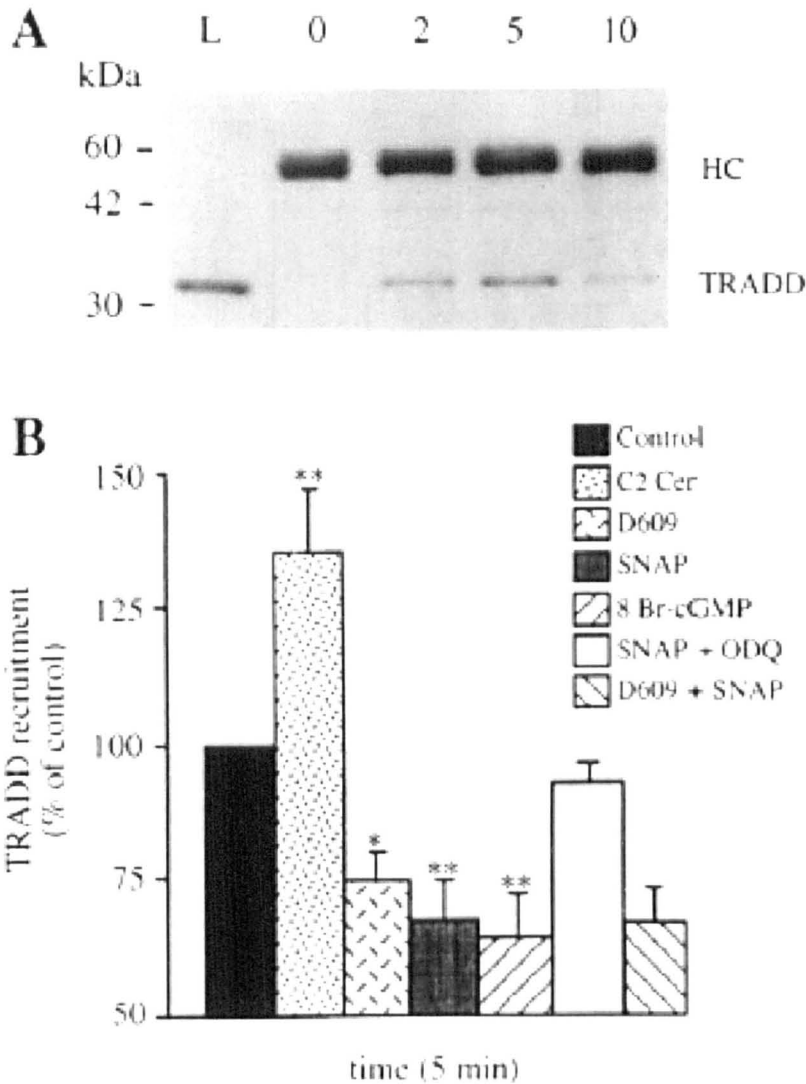


Figure 1.15) Effects of ceramide, NO, and cGMP on TNF- α -induced TRADD recruitment to TNF-R1.

- A)** Time course of TRADD recruitment induced by TNF- α (50 ng/ml)/CHX (1 μ g/ml) revealed by Western blotting of the precipitates with an anti-TRADD Ab. The gel shown is representative of four individual experiments: 0, 2, 5, and 10 indicate the time (min) of cell exposure to TNF- α before immunoprecipitation. Thirty micrograms of whole-cell homogenate was loaded in lane L as an internal control. Migration of the Ig heavy chain (HC) and TRADD is specified on the right.
- B)** Cells were treated with TNF- α (50 ng/ml)/CHX (1 μ g/ml) (**control**) with or without **SNAP** (300 μ M), **8 Br-cGMP** (3 mM), **ODQ** (1 μ M), **D609** (25 μ g/ml) and **C2 Cer** (20 μ M) in various combinations, as indicated in the key. The specific bands recognized by the anti-TRADD Ab were analyzed by densitometry, and values are expressed as a percentage of those observed in the TNF- α /CHX-treated control ($n=5$).

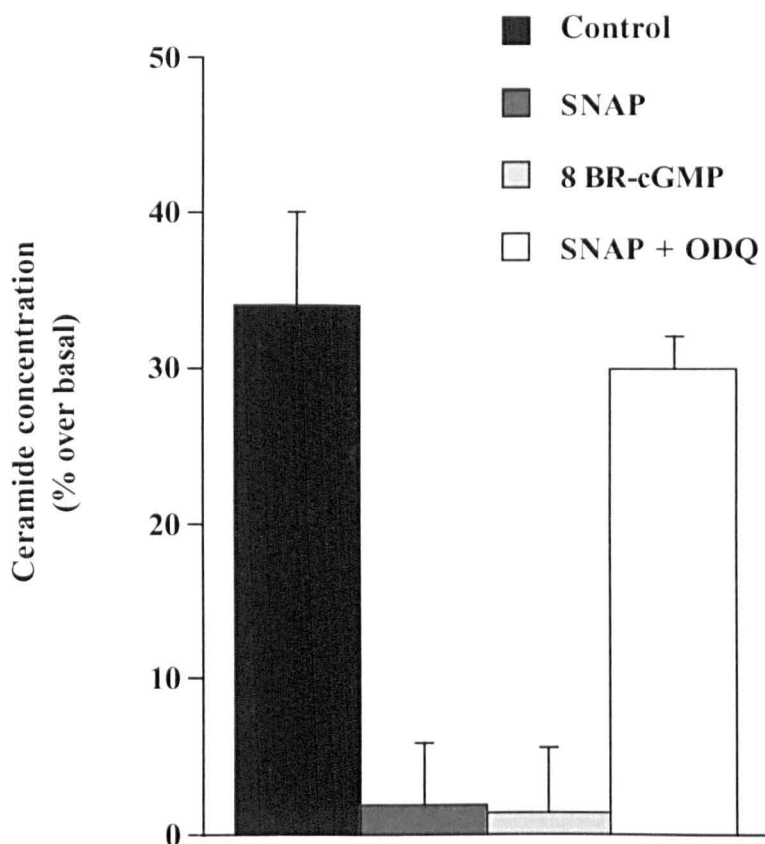


Figure 1.16) Effects of NO and cGMP on TNF- α -induced ceramide generation.

Cells were treated with TNF- α (50 ng/ml)/CHX (1 μ g/ml) (**control**) with or without **SNAP** (300 μ M), **8 Br-cGMP** (3 mM), and **ODQ** (1 μ M) as indicated in the key. Cell aliquots were collected and lysed, and ceramide content was measured by DAG kinase assay as described in (DeNadai et al, 2000). Ceramide concentrations are expressed as a percentage of those measured in cell samples run in parallel but not treated with TNF- α /CHX (basal value were 140 – 8 pmol/mg proteins) ($n=5$).

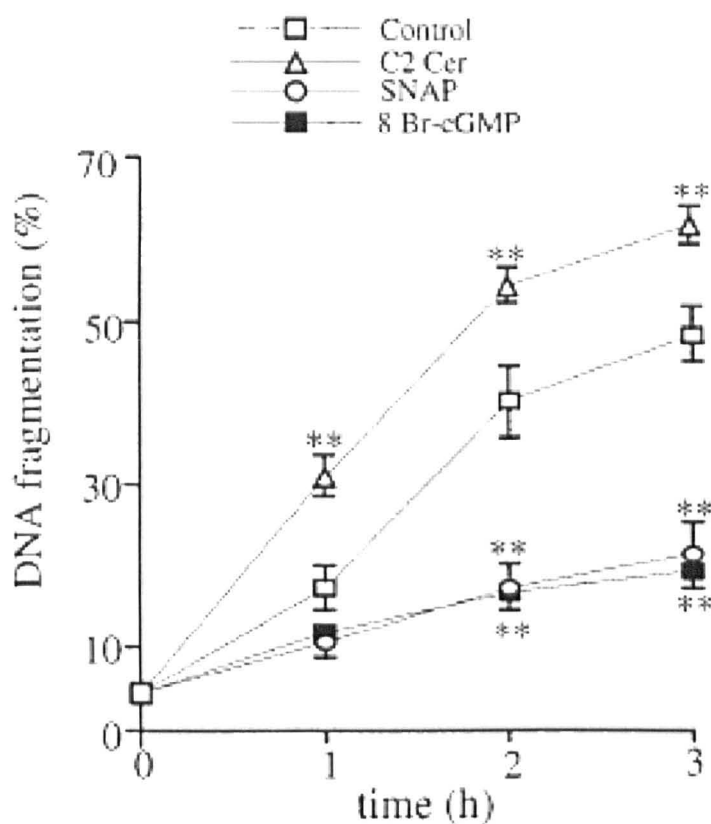


Fig. 1.17) Effects of exogenous ceramide, NO, and cGMP on TNF- α -induced apoptosis.

U937 Cells were treated with TNF- α (50 ng/ml)/CHX (1 μ g/ml) (**control**) with or without **SNAP** (300 μ M), **8 Br-cGMP** (3 mM), and **C2 Cer** (20 μ M) for the indicated time points before DNA fragmentation analysis as indicated in (**DeNadai et al, 2000**)

Chapter 2

RESULTS

Effect of exogenous NO on early signalling by TNF- α in U937 cells

2.1) Time course of TNF- α -triggered sphingomyelinase activation

In order to elucidate the enzymatic activity(ies) responsible for the early wave of ceramide accumulation in the cell as showed in our previous paper (De Nadai et al, 2000), I measured the activation of both N- and A-SMase in U937 cells at different time points after TNF- α stimulation, taking advantage of the method first described by Wiegmann et al, (1994). This method is based on the measurement of the in vitro conversion of radioactively labelled sphingomyelin substrate, as described in the *materials and methods* section.

U937 cells were treated with 50 ng/ml TNF- α for the time points indicated in **Figure 2.1**, and the SMase activity of cell lysates was measured at the optimum pH for either enzymes (pH 5.5 for A-SMase and pH 7.4 for N-SMase). SMase activity was defined as the percentage of radio-labelled sphingomyelin that was converted to its degradation product, phosphoryl-choline, evaluated as the radioactivity collected in the aqueous phase after the separation from organic solvents, and measured by liquid scintillation.

As shown in **Figure 2.1**, TNF- α treatment caused a very rapid activation of both SMase, that was clearly detected two minutes after treatment, peaked after 5 minutes and then levelled off, returning to the baseline values after 15 —30 minutes.

No further increase of the A-SMase activity was detected during further incubations (**Fig 2.1A**), while N-SMase had a second prolonged phase of rise, starting after 90 minutes of the stimulation with the cytokine and continuing to increase for the rest of the observation period (**Fig2.1B**). The reason for this delayed N-SMase activation is unknown, but a possibility is that the fall of GSH levels, reported to happen in these cells after TNF- α treatment, has a role in this result (Higuchi et al, 1998).

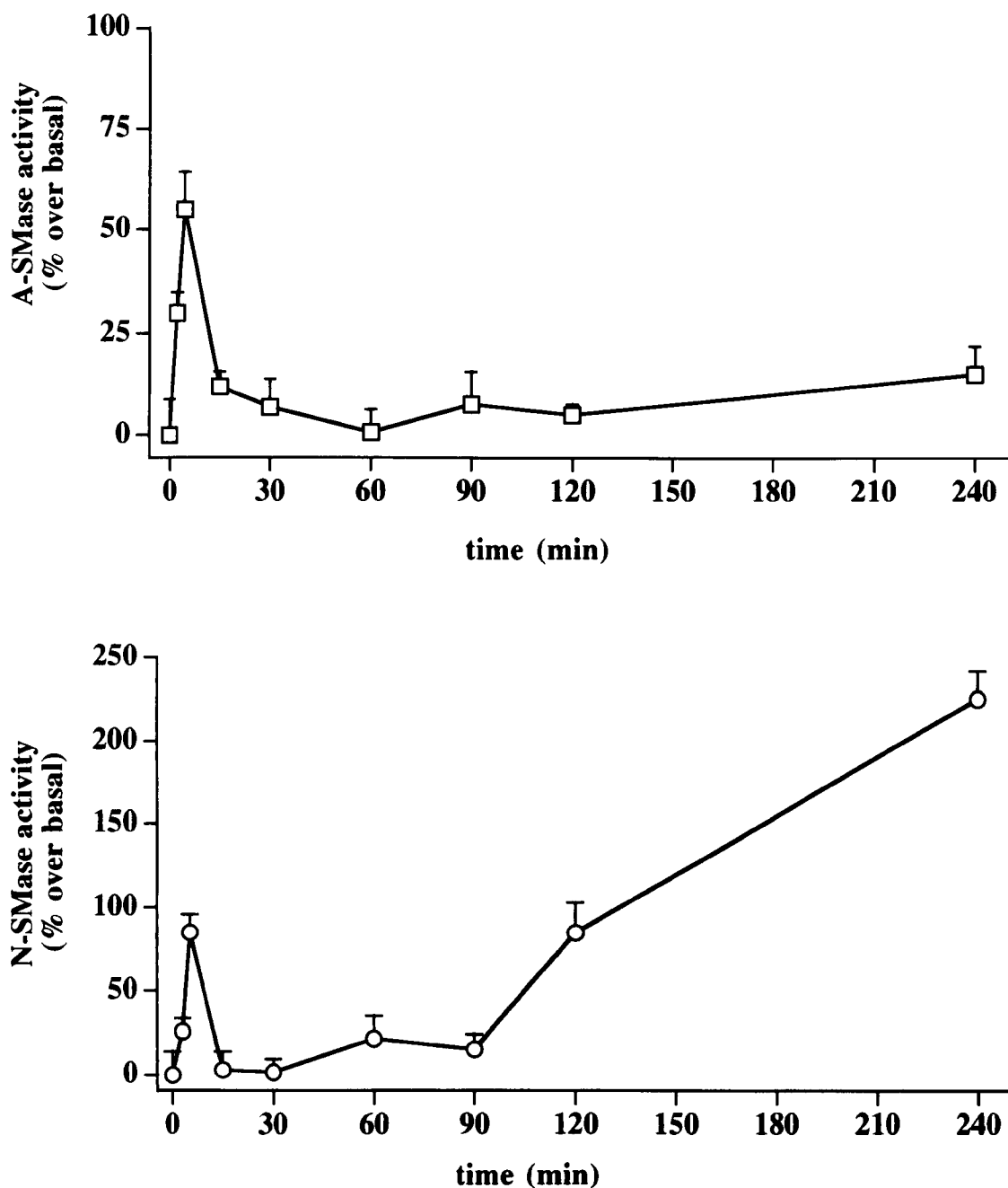


Figure 2.1) Time course of activation of A-SMase (A) and N-SMase (B) by TNF- α in U937 cells

Cells were treated with 50 ng/ml TNF- α and samples were collected at the indicated time points. SMase activities were determined in cell lysates by measuring sphingomyelin hydrolysis to phosphoryl-choline at pH 5.5 (A) and 7.4 (B), the lysis buffers were specific for the pertinent SMase to test (as indicated in the Materials and Method section).

Values are expressed as % \pm s.e.m over basal SMase activity (Values were 21 ± 5 pmol/mg min^{-1} for A-SMase and 9 ± 4 pmol/mg min^{-1} for N-SMase) (n=5)

2.2) NO inhibits sphingomyelinases activation

Since both SMases were found to be activated in the first minutes following TNF- α treatment, and since, in our previous paper NO was able to induce protection from TNF- α induced apoptosis in these cells in the first minutes following the cytokine administration, I decided to analyze the effect of NO on the activation of both SMase induced by TNF- α in the peaking activity time window. Specifically, I measured the peak activation of both SMases showed on **Figure 2.1**, that is after five minutes of TNF- α , in the presence of increasing concentration of the NO donor SNAP, and I used concentration of SNAP from 0 to 300 μ M which had no cytotoxic effect (**DeNadai et al, 2000**)

Results are shown on **Figure 2.2A**: both A- and N-SMase activation were progressively inhibited by increasing concentration of SNAP, albeit with different apparent IC_{50} for the two activities: A-SMase responded to a lower concentration of SNAP (apparent IC_{50} 10 μ M), while N-SMase inhibition required a higher concentration of NO (apparent IC_{50} 45 μ M).

I then explored whether this effect of NO was direct, or whether it involved the activation of soluble guanylate cyclase and the formation of cGMP. To this end I exposed the cells to TNF- α in the presence of the cell permeant cGMP analogue, 8 Br-cGMP (1 mM), and the compound BAY 41-2272 (1 μ M), which is able to stimulate soluble GC through a NO-independent site (**Stasch et al, 2001**), mimicking its action. Under these conditions the increased activities of both SMases following TNF- α treatment were inhibited.

The inhibition of sGC by the specific inhibitor H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 1 μ M) (**Moncada and Higgs, 1995**) was able to reverse the inhibitory effect of SNAP (300 μ M) on both A- and N-SMase; the same effect was induced by the pretreatment of the cells with the protein kinase G inhibitor, KT5823 (**Gadbois et al, 1992**). All these effects are displayed on **figure 2.2B**. Basal sphingomyelin hydrolysis was never affected in the SMase assay when all these compounds were administered in the absence of TNF- α .

Taken together, these results, obtained with various and structurally unrelated pharmacological tools, indicate that NO is able to inhibit the TNF- α triggered A- and N-SMase activation via a mechanism involving the activation of GC, formation of cGMP and activation of protein kinase G

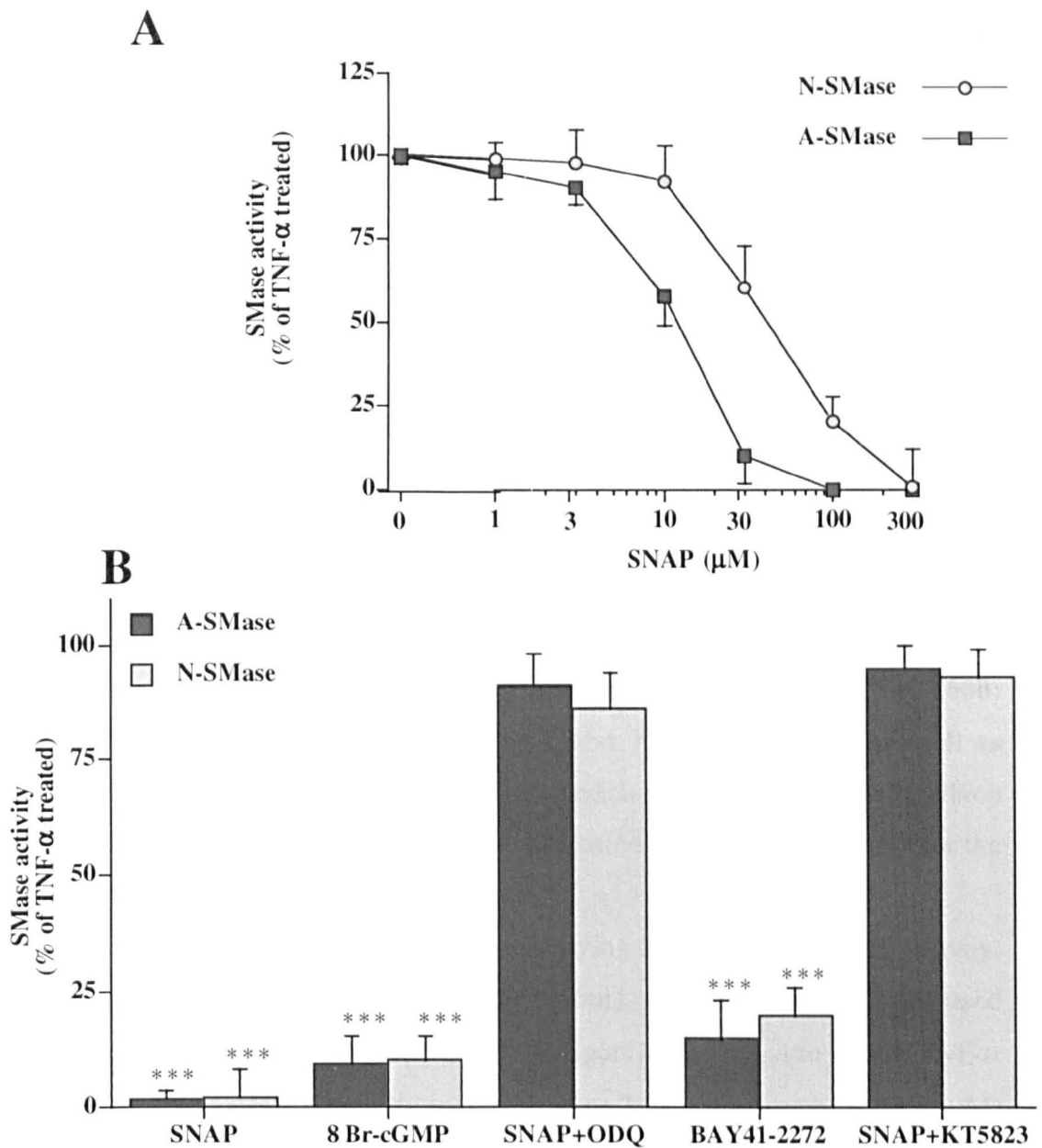


Figure 2.2) NO inhibits TNF- α triggered A-SMase and N-SMase activities through the generation of cGMP and the activation of Protein Kinase G

A) U937 cells were treated with 5 minutes **TNF- α** (50 ng/ml) in the presence of increasing concentrations of the exogenous NO donor, **SNAP** (0-300 μ M)

B) Cells were treated for 5 minutes with **TNF- α** (50 ng/ml) with or without **SNAP** (300 μ M), **8Br-cGMP** (1 mM), **SNAP + ODQ** (1 μ M), **BAY-41-2272** (1 μ M) and **SNAP** (300 μ M) + **KT5823** (1 μ M).

In both the panels SMase activities were measured as described in Fig 2.1 legend and expressed as % – s.e.m. of the activities measured in the presence of only TNF- α (considered as 100 %: see **figure 2.1** legend for absolute values). Statistical values are measured vs cells treated with TNF- α alone (n=6)

2.3) Contribution of sphingomyelinases to early features of apoptosis

The contribution of the various forms of SMase activities to apoptosis has been found to vary depending on the cell system utilized (see Introduction section and the following refs (Perry and Hannun, 1998; Levade and Jaffrezou, 1999; Lozano et al, 2001; Segui et al, 2001). Till now, however, no knowledge is available about the possible role and contribution of each enzyme to the early signalling of TNF- α .

2.3a) Specificity of sphingomyelinases inhibitors

To try to evaluate the specificity problem we used a range of known compounds, namely imipramine (Jensen et al, 1999) tricyclodecan-9-yl xanthate (D609, DeNadai et al, 2000) and monensin (Garcia-Ruiz et al, 2000), which inhibit A-SMase activity, as well as scyphostatine (Tanaka et al, 1997) and L-carnitine (Andrieu-Abadie et al, 1999), which are inhibitors of N-SMase activity. Cells were pre-incubated with these compound for the times indicated in the Materials and Method section.

The specificity of the compounds was checked by assaying SMases at their peak activity, i.e. after 5 minutes treatment with TNF- α (50 ng/ml). At the concentrations used imipramine (20 μ M), monensin (1 μ M) and D609 (25 μ g/ml) were found to inhibit TNF- α triggered A-SMase, and not N-SMase activity (see **Figure 2.3**), while scyphostatin (1 μ M) and L-carnitine (10 μ g/ml) inhibited N-SMase, and not A-SMase, activity (see **figure 2.4**) triggered by TNF- α .

Of importance, when these compounds were administered alone they didn't have any effect on the basal conversion of sphingomyelin to ceramide: for A-SMase absolute values were imi 19–4 pmol/mg min⁻¹; mon 18–5 pmol/mg min⁻¹; D609 20–3 pmol/mg min⁻¹; L-carn 22–5 pmol/mg min⁻¹; scypho 20–2 pmol/mg min⁻¹; for N-SMase absolute values were imi 9–4 pmol/mg min⁻¹; mon 8–5 pmol/mg min⁻¹; D609 8–3 pmol/mg min⁻¹; L-carn 9–2 pmol/mg min⁻¹; scypho 8–2 pmol/mg min⁻¹.

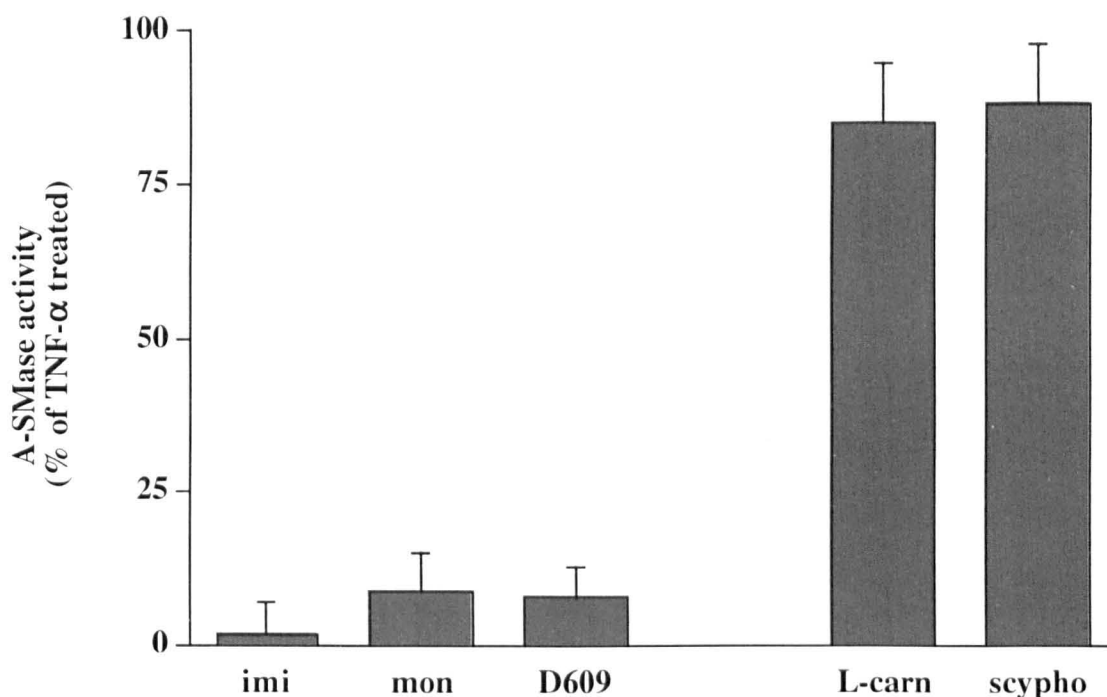


Figure 2.3) Specificity of action of drugs on **A-SMase** activity triggered by **TNF- α**

U937 cells were treated for 5 minutes with **TNF- α** (50 ng/ml) with or without imipramine (**imi**, 20 μ M), monensin (**mon**, 1 μ M), D609 (25 μ g/ml), L-carnitine (**L-carn.** 10 μ g/ml) and scyphostatin (**scypho** 1 μ M). A-SMase activity was measured as described in the Fig 2.1 legend and expressed again as % – s.e.m. of the activity measured in presence of only **TNF- α** alone (considered as 100%). Absolute values were: untreated cells 21–5 pmol/mg min⁻¹; cells treated with **TNF- α** 33–4 pmol/mg min⁻¹.

In the present figure I only show the values obtained by co-incubation of the various compound with **TNF- α** , as the incubation with only the compounds doesn't result in any variation from untreated cells in terms of spingomyelin conversion (see text at pag 60 for absolute values).

Indicated statistical values were measured versus cells treated with **TNF- α** alone (n=5)

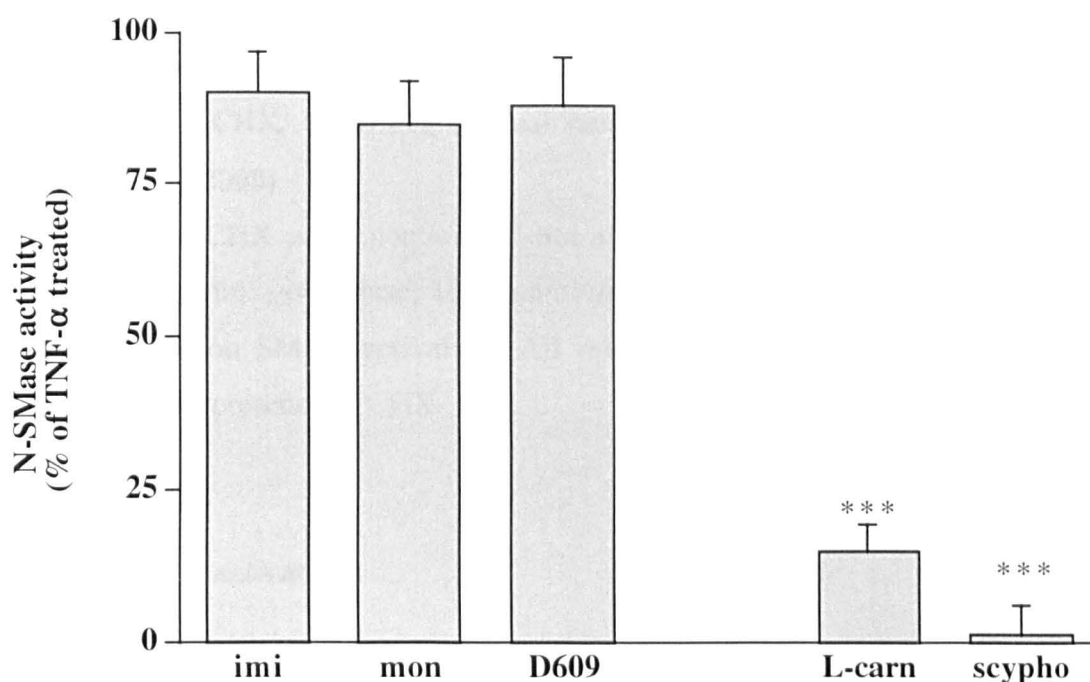


Figure 2.4) Specificity of action of drugs on **N-SMase** activity triggered by TNF- α

U937 cells were treated for 5 minutes with TNF- α (50 ng/ml) with or without imipramine (**imi**, 20 μ M), monensin (**mon**, 1 μ M), D609 (**D609**, 25 μ g/ml), L-carnitine (**L-carn.** 10 μ g/ml) and scyphostatin (**scypho** 1 μ M). A-SMase activity was measured as described in the Fig 2.1 legend and expressed again as % \pm s.e.m. of the activity measured in presence of only TNF- α alone (considered as 100%). Absolute values were: untreated cells 9 ± 4 pmol/mg min $^{-1}$; cells treated with TNF- α : 17 ± 3 pmol/mg min $^{-1}$.

Again I only show the co-incubation of the various compounds with TNF- α , as the incubation with only the drugs doesn't result in any variation from untreated cells (see text at page 60 for absolute values).

Indicated statistical values were measured versus cells treated with TNF- α alone (n=5)

I have consequently used the previously described compounds to pharmacologically dissect the contribution of the different SMase activities to the initial phase of apoptosis triggered by the administration of TNF- α in the presence of the protein synthesis inhibitor cycloheximide (CHX, 1 μ g/ml), a usual paradigm for studying apoptosis by TNF- α (DeNadai et al, 2000).

Of importance, CHX preincubation did not alter the SM conversion profile (A-SMase: 20–4 pmol/mg min⁻¹; N-SMase: 10 \pm 3 pmol/mg min⁻¹) ruling out a possible interference effect of CHX on SMase activation. All the subsequent experiments were therefore performed in the presence of CHX.

2.3b) Caspase-8 activation

We have analyzed another initial hallmark of apoptotic signalling by TNF- α , that is the activation of caspase-8, which was measured by two different techniques.

Cleavage of a specific fluorescent substrate, N-acetyl-IETDE-7-amino-4-trifluoromethylcoumarin, was measured with the fluorimeter. Basal activity of caspase 8 was 10 \pm 1 pmol/mg min⁻¹ of converted specific substrate, and rose 75% after 5 minutes of treatment with TNF- α /CHX. The pre-treatment of the cells with only the various SMase pertinent inhibitors didn't alter the basal level of caspase-8 activity, ruling out any effect of the drugs on this feature, at least in the considered time frame.

The TNF- α stimulated increase of caspase-8 activity was specifically inhibited by preincubation of the cells with imipramine, D609 and with monensin, while neither scyphostatine nor L-carnitine had any relevant and significant effect on this parameter (Figure 2.5).

Consistent with this result, A-SMase inhibitors prevented the reduction in the amount of the uncleaved and inactive procaspase-8 by TNF- α with respect to the control, as indicated by western blot experiments of Figure 2.5B. As an internal control I measured cleavage of procaspase-3, which get cleaved later during apoptosis progression. Caspase-3 was not affected by the treatments, as shown by relevant band in the western blot of Figure 2.6B.

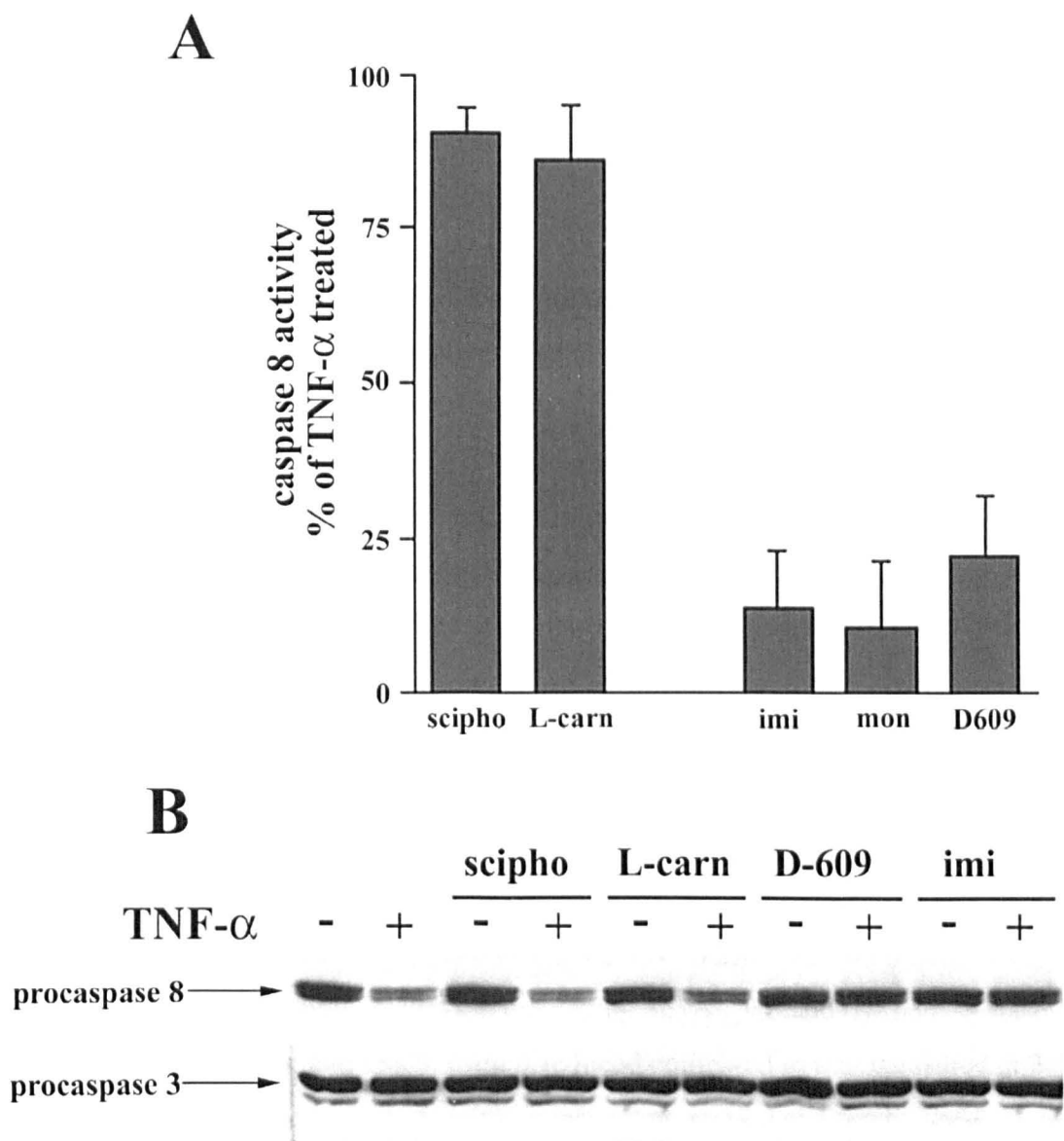


Figure 2.5) Involvement of A-SMase in caspase-8 activation triggered by TNF- α /CHX.

U937 cells were treated with **TNF- α** (50 ng/ml) in the presence of the indicated compounds (described in the previous figure legend and in the text). Caspase 8 activity was measured after 5 minutes by two independent mean: fluorimetric measure of a caspase-8 specific substrate (**A**) and Western Blot analysis (**B**, upper panel) of proteolysis of the inactive zymogen, procaspase8. none of the treatment results in proteolysis of procaspase-3 (**B**, lower panel), a downstream effector caspase, used in this case as an internal control.

2.3c) DNA fragmentation

I then evaluated the effects of SMases on two more 'downstream' steps of apoptosis: DNA fragmentation and phosphatidyl-serine exposure to the outer leaflet of the plasma membrane.

DNA fragmentation was evaluated by the established method of filter binding assay (DeNadai et al, 2000). We checked the amount of fragmented DNA at various time points (1, 2 and 3 hours) after TNF- α /CHX treatment in the presence of the various SMase inhibitors. As showed in **Figure 2.6**, the pre-treatment with A-SMase inhibitors resulted in a considerable inhibition of DNA fragmentation in the 3 h time window, while N-SMase inhibitors didn't alter in any way the profile of fragmentation.

The pretreatment of the cells with the various compounds, in the absence of TNF- α stimuli, didn't cause any alteration of DNA fragmentation, with the percentage of fragmented DNA that wasn't different from the basal level of 5% (see **figure 2.6**).

We next investigated whether the early mechanism of drug protection has any role also in the progression of the apoptotic process, by measuring the kinetics of DNA fragmentation after the 3 hours time window: **Table 2.1** shows this parameter after 4.5 and 6 hours after administration of the death stimuli. Protection exerted by 8Br-cCMP and by the A-SMase activity inhibitors, imipramine and D609, even if reduced compared to that measured at 3 hours, was still significant after 4.5 hr. At 6 hours instead protection exerted by these compounds was not detected anymore.

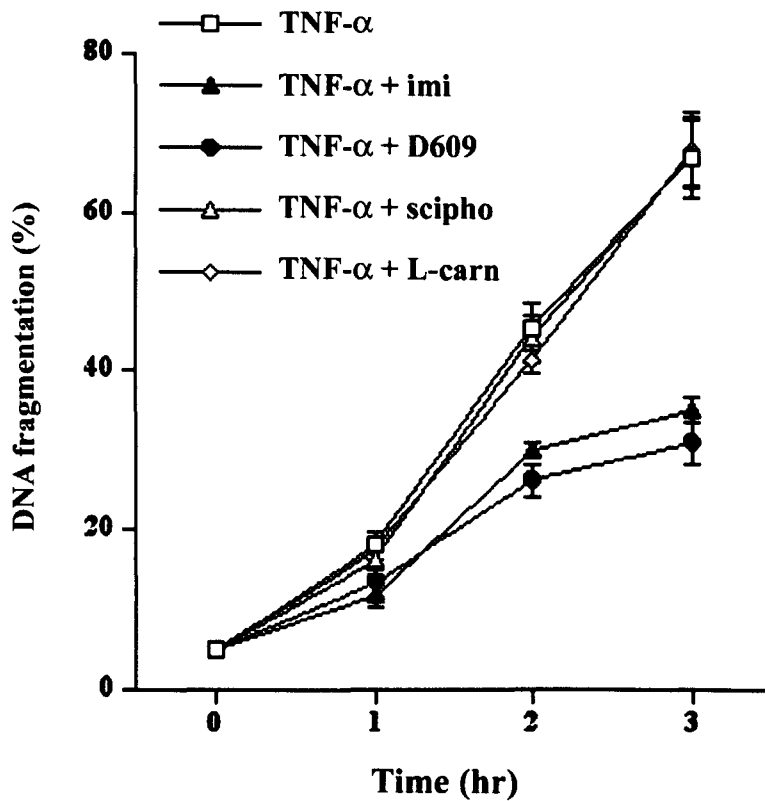


Figure 2.6) Involvement of A-SMase in DNA fragmentation triggered by TNF- α /CHX

U937 cells were treated with TNF- α /CHX (50 ng/ml) in the presence of the already described drugs and DNA fragmentation, evaluated by the filter binding assay, was analysed at the indicated times after cytokine stimuli indicated in the X axis.

Table 2.1) Inhibition by cGMP and potentiation by A-SMase of TNF- α -induced apoptosis are transient events

time (h)	DNA fragmentation (%)		
	3	4.5	6
TNF- α	65 \pm 5	82 \pm 5	94 \pm 4.
TNF- α + 8 Br-cGMP	27 \pm 2***	59.5 \pm 3**	88 \pm 5
TNF- α + imi	33 \pm 5***	62 \pm 4**	96 \pm 6
TNF- α + D609	31 \pm 2***	64 \pm 5*	93 \pm 5

U937 cells were treated with TNF- α (50 ng/ml)/CHX (1 μ g/ml) with or without 8 Br-cGMP (1 mM), imipramine (imi, 20 μ M) or D609 (25 μ g/ml). DNA fragmentation was measured after 3, 4.5 and 6 h of incubation with the cytokine, as detailed in the Materials and Methods section. Values are expressed as % \pm s.e.m. Statistical values in the various samples are measured vs. cells treated with TNF- α /CHX ($n = 8$).

2.3d) Phosphatidyl-serine exposure

Phosphatidyl-serine exposure to the outer leaflet of the plasma membrane occurs early during apoptosis, and can be measured by cyto-fluorimetric analysis of fluorescein-labelled annexinV. The latter protein specifically interacts with phosphatidyl-serine residues. I have measured the appearance of annexinV-positive cells after three hours of treatment with TNF- α , testing, again, the role of each SMase by the use of the above mentioned A- and N-SMase inhibitors.

I found that the preincubation of the cells with A-SMase specific inhibitors caused a minor positivity of the cells to annexinV staining, (see **Figure 2.7**), while pre-treatment with only N-SMase inhibitors didn't have any relevant effect on the same parameter. As in the other previous experimental settings, pre-treatment of the cells with these compounds alone didn't have any consequence in term of apoptotic marker staining.

Taken together these results indicates that A-SMase contributes to early apoptotic features of TNF- α signalling, while N-SMase seems not to be involved at least in this stage of the apoptotic process.

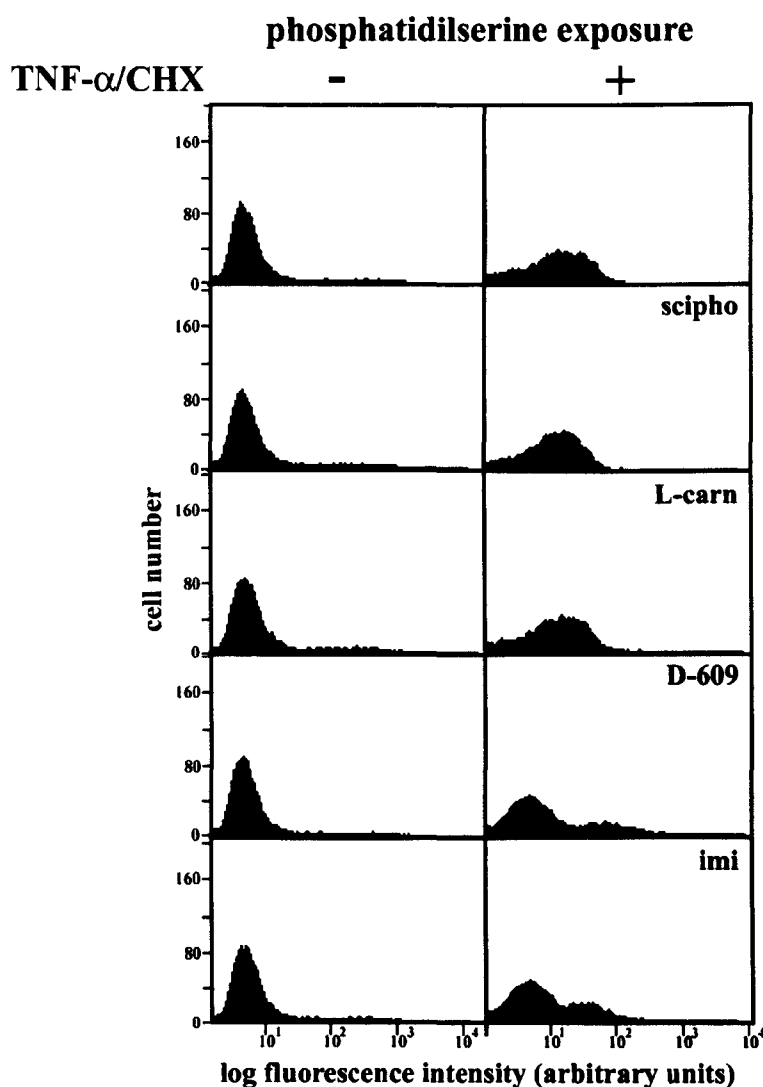


Figure 2.7) Involvement of A-SMase in phosphatidyl-serine exposure triggered by TNF- α /CHX

U937 cells were treated with TNF- α /CHX (50 ng/ml) in the presence or absence of indicated compounds, and annexinV staining was analysed at the cytofluorimeter after three hours of incubation with the cytokine. The annexin V staining profile of untreated cells is super-imposable to that of unstained cells.

2.4) cGMP dependent inhibition of acidic-sphingomyelinase accounts for apoptosis protection

I then investigated whether the cGMP dependent inhibition of A-SMase activity shown in **Figure 2.2** could account for the NO/cGMP dependent protection from TNF- α -induced early apoptosis that was described by **DeNadai et al (2000)**. To this end I have examined directly the effects of the cyclic nucleotide.

As shown in **Figure 2.8** administration of 8Br-cGMP inhibited both caspase 8 activation (**Figure 2.8A**) and DNA fragmentation (evaluated at the 3 hours time point; **Figure 2.8B**) triggered by TNF- α (5 minutes). When the cyclic nucleotide was administered together with imipramine, monensin or D609, neither caspase8 activation nor DNA fragmentation were inhibited further. The lack of a synergistic effect on the apoptotic feature indicates that the inhibition by the two applied agents occurs at the same signalling event. On the other hand, the administration of the long chain natural C16 ceramide (30 μ M) reversed the effects of 8Br-cGMP and A-SMase inhibitors on both TNF- α /CHX triggered caspase8 activation and DNA fragmentation.

When administered alone, and at the concentrations and for the time window employed, C16 had effect neither on caspase8 activation or DNA fragmentation, indicating that ceramide action is exerted only when the apoptotic signalling is activated by TNF- α treatment, that is upon DISC complex formation.

Taken together, the data presented in section 2.1-4 strongly indicate that NO is able to inhibit both A- and N-SMase through a pathway involving generation of cGMP and activation of the protein kinase G, which had not yet been considered. Inhibition of the A-SMase and of the ensuing generation of ceramide seems to account for the cGMP dependent, early inhibitory effect of NO on TNF- α -induced apoptosis. The results obtained measuring level of DNA fragmentation after 6 hours show that inhibition of A-SMase by NO/cGMP delays, rather than totally preventing, the TNF- α /CHX induced apoptosis. Thus, modulation of A-SMase by NO/cGMP acts as a regulatory mechanism rather than a switch off system.

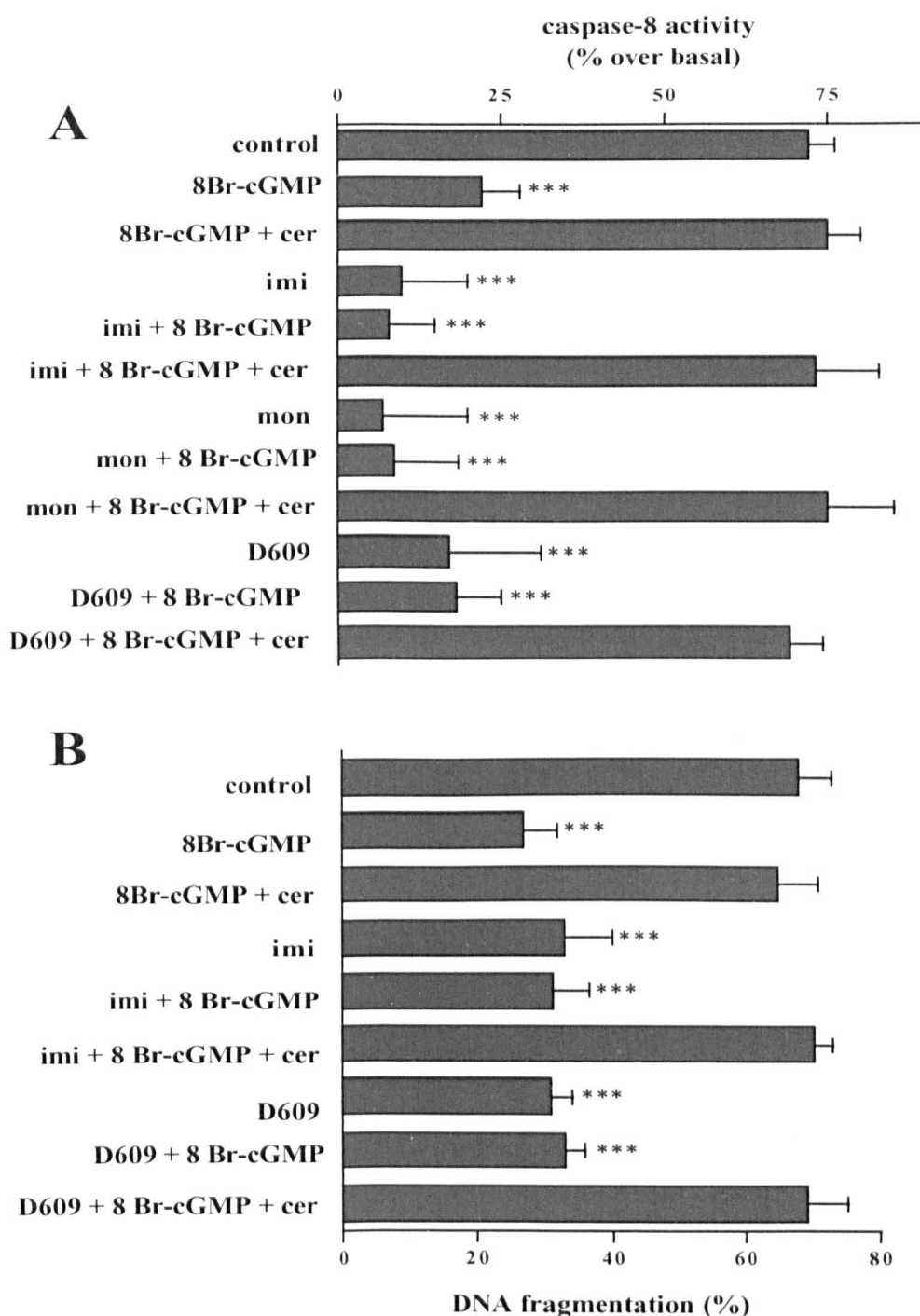


Figure 2.8) Inhibition of A-SMase by 8Br-cGMP accounts for its ability to reduce TNF- α /CHX induced caspase-8 activation and DNA fragmentation

All treatments showed in the figure were carried out as described in the previous legends and in the text; C16 ceramide concentration was 30 μ M (**cer**). Treatments were carried out in various combinations as indicated in the y axis. Caspase 8 activity (**A**) was measured after 5 minutes, while DNA fragmentation (**B**) after 3 hours of incubation with the cytokine. Control in each panel represents caspase activity (absolute values for caspase-8 activity: untreated cells 10 ± 1 pmol/mg min⁻¹; TNF- α treated cells 18 ± 1 pmol/mg min⁻¹) or DNA fragmentation induced by TNF- α /CHX alone (n=5). In both panels statistical values are measured vs cells treated with TNF- α /CHX (**control**) (n=6)

Effect of endogenous NO on TNF- α induced cell death in HeLa/NOS tet off cells

2.5) Construction and characterization of HeLa/eNOS tet-off cells

2.5a) Transfection and Western blot

In order to analyse the possible effects of endogenous NO on cell death signalling triggered by TNF- α , we decided to build up a system where eNOS expression could be conditionally induced. To this end, we used the Tet-off system, which, by allowing comparison between the 'switched on' and 'switched off' state of transcription of the transfected cDNA, permits direct assessment of the consequences of expression of the considered gene in an internally controlled and regulated system.

Experiments were conducted with HeLa tet off cells commercially available from CLONTECH, made stably transfected with a bovine eNOS cDNA (kind gift of William C. Sessa), put under the control of a tetracycline repressed promoter. The tet off system (Gossen and Bujard, 1992) allows controlled expression of a single gene by the presence/absence in the culture medium of tetracycline or of its derivative doxycycline.

This represents therefore a precious tool to study the direct effect of the expression of eNOS in the context of TNF- α signalling, since it permits to compare cells originally not endowed with the enzyme with cells where eNOS has been forced to be expressed.

We proceeded with transfection of HeLa Tet-off cells with eNOS cDNA cloned in the p-TRE plasmid by CLONTECH, together with a plasmid conferring resistance to hygromycin. 30 hygromycin resistant clones were obtained and expanded, of which 6 expressed eNOS when doxycycline was removed from the culture medium: this was established by western blotting and immuno-fluorescence. I carried out a detailed characterization on one of the selected clones, which was named clone A δ .

I analyzed the time course of induction of expression of eNOS by doxycycline removal from the culture medium as seen in **Figure 2.9A**: the gel lanes were loaded with 10 μ g of lysates prepared from cells cultured for the indicated time in absence of doxycycline. Removal of doxycycline did cause the rapid induction of eNOS expression, detected as a 140000 Mr band using a monoclonal anti-eNOS antibody. The relevant band was already present 4-6 hours after removal of doxycycline, and continued to increase until 72 hours,

when expression reached a steady state. Equal protein loading was internally controlled by β -actin staining on the same nitrocellulose filters.

I also investigated the dependency of eNOS levels on doxycycline concentration as reported in **Figure 2.9B**: induction was detectable at doxycycline concentration less than 0.1 ng/ml. With decreasing doxycycline concentration eNOS expression was progressively increased, reaching its maximum in the absence of the antibiotic. In all the cases we checked eNOS expression after 72 hours of doxycycline removal from the medium. The level of eNOS in HeLa A δ after 72 hours of induction was comparable to the endogenous eNOS present in an endothelial cell line (E2 cells, a kind gift of Elisabetta Dejana).

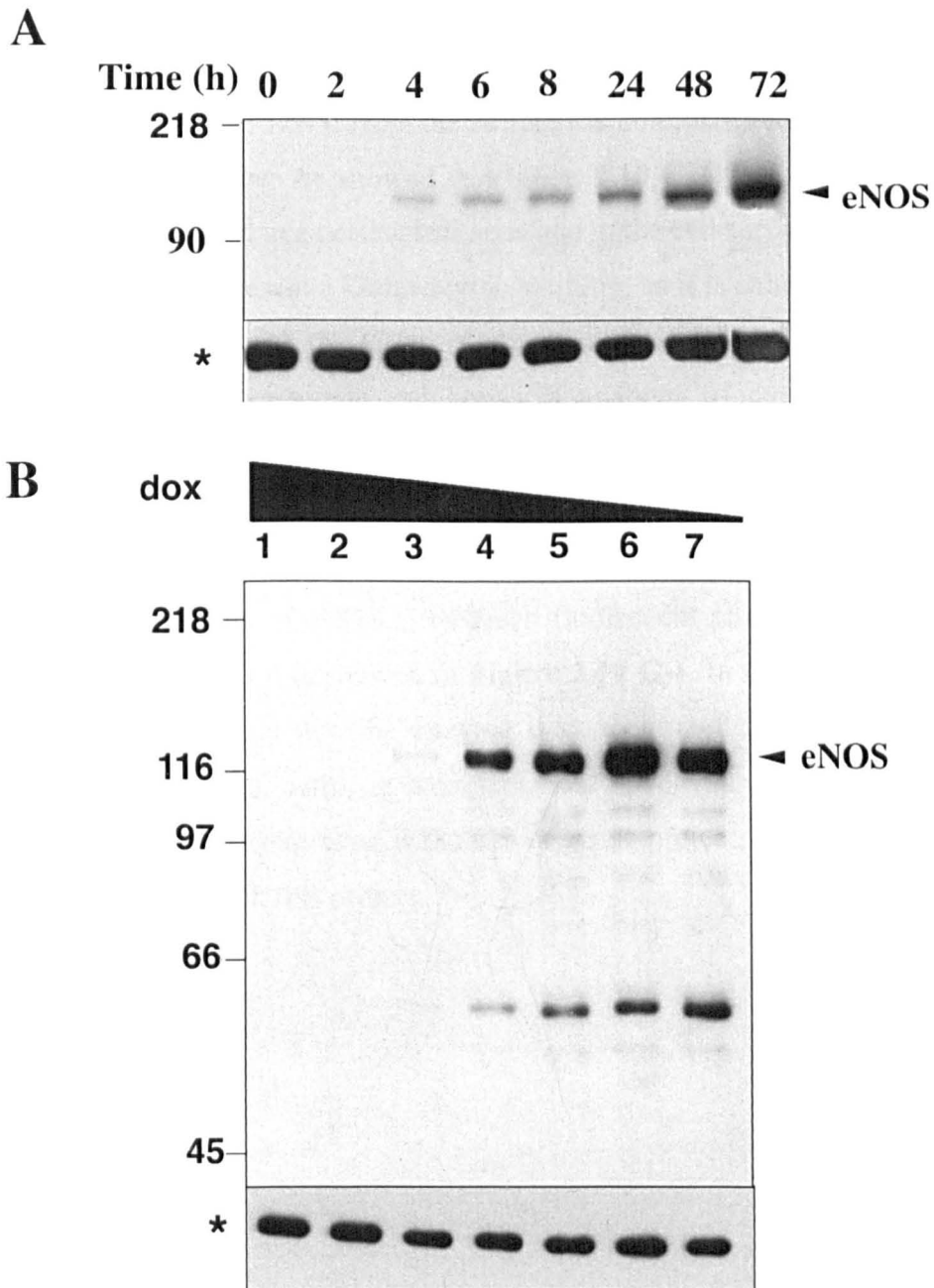


Figure 2.9) Immunoblots of proteins from HeLa tet-off cells.

- A)** Cells were grown to confluence in the presence of doxycycline, then split and incubated in smaller dishes in the absence of doxycycline for the indicated time points.
- B)** Cells were cultured as above, but, after being split, they were cultured in the presence of 1, 0.5, 0.1, 0.05, 0.01, 0.001 and 0 ng/ml doxycycline for 72 hours (lanes 1-7)

Cell lysates were subjected to SDS-PAGE and the resulting nitrocellulose filters were probed with either an anti-eNOS (arrowhead, upper panels of A and B) or with an anti- β -actin antibody (asterisks, lower panel of A and B)

2.5b) Sub-cellular localisation of eNOS

By means of immunocytochemistry analysis we then investigated whether the enzyme, expressed after doxycycline removal from the culture medium, has a localisation similar to that described in the literature. As showed in **Figure 2.10** eNOS immunoreactivity was found to be most concentrated in a perinuclear zone and at the cell surface.

The perinuclear staining represent a Golgi region staining, as it is showed in **Fig 2.10A-G** by colocalisation of eNOS with the Golgi marker giantin in the same fields of cells in double immunostaining experiments and confocal analysis of the specimen. This is consistent with reports in the literature about localisation of the native enzyme in endothelial cells (**Sessa et al, 1995**).

We have also performed a close examination of eNOS surface distribution by double immunostaining experiments of eNOS with green fluorescent phalloidotoxin, a specific label for cytoskeletal actin as it is showed in **Figure 2.10 G-I**. In these series of confocal images it is well recognisable that the enzyme is concentrated in areas of the plasma membrane enriched in cortical actin, an interesting and novel finding which could have a functional meaning, whose exploitation is the aim of a side project. I won t describe in this thesis the results obtained with this project.

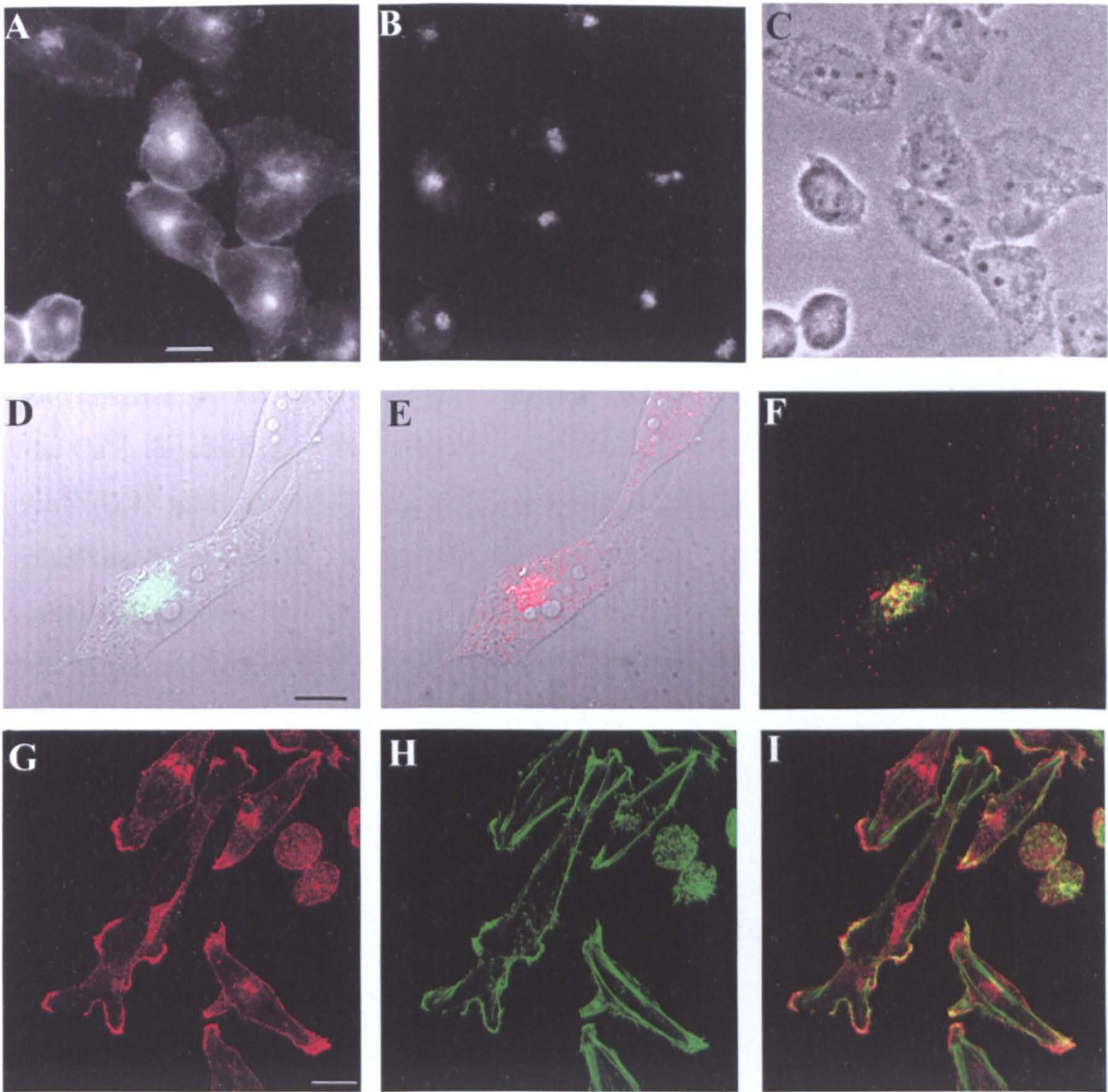


Figure 2.10) Double-staining immunofluorescence of HeLa tet-off cells

A-F) Cells expressing eNOS were doubly stained with monoclonal anti-eNOS and polyclonal anti-giantin antibodies, followed by fluorescein-labeled anti-mouse IgG (**A** and **D**) and rhodamine-conjugated anti-rabbit IgG (**B** and **E**). *Panel A-C* shows the same field of cells viewed at the fluorescent microscope under the fluorescein filter for eNOS (**A**), the rhodamine filter for giantin (**B**) and in phase contrast (**C**): eNOS appears concentrated on the surface and in perinuclear structure corresponding to the Golgi region, as shown by the giantin staining (**B**). *Panel D-F* shows a confocal microscopy analysis of a single cell: eNOS (**D**) and giantin (**E**) images are superimposed to the transmitted light image, and co-localization of the two is showed by the yellow color in the merged image (**F**)

G-L) Cells expressing eNOS were stained with monoclonal anti-eNOS antibody followed by rhodamine-conjugated anti-mouse IgG (**G**) and with fluorescein-labelled phalloidotoxin (**H**). the merged images (**I**) show co-localization of eNOS with cortical actin, as demonstrated by the appearance of yellow colour.

Bar in panels A-C: 15 μm ; panels D-F: 10 μm ; panels G-I: 20 μm

2.5c) Transfected eNOS activity and cGMP assays.

To functionally characterize the transfected HeLa cell line in terms of eNOS activity and NO generation, I measured the Ca^{2+} dependent eNOS activity by assaying the conversion of the radiolabelled, L-[^3H]-arginine into the reaction product, L-citrulline, produced in a stoichiometrical quantity to NO.

These assays were carried out on cell lysates of cells cultured in presence (NOS negative) and absence (NOS positive) of doxycycline. Various conditions were employed to evaluate the Ca^{2+} dependency of the enzyme: in the presence of Ca^{2+} (0.45 mM), in the presence of Ca^{2+} (0.45 mM) and the calcium chelator EGTA, and in the presence of Ca^{2+} (0.45 mM) plus the NOS inhibitor L-nitro mono-methyl arginine (NMMA, 0.5mM). Radioactive labelled substrate and product of the reaction were then separated by mean of ionic exchange cromatography as first showed by **Bredt and Snyder (1989)**, using the strong cationic exchange resin Dowex 50X8-400.

Results are shown in **Figure 2.11A**. Citrulline formation in eNOS expressing (**dox-**) cells was stimulated by the presence of Ca^{2+} to a level, 2.5 pmol/mg min⁻¹, comparable with those reported in the literature for endothelial cells (see e.g. **Shaul et al, 1996**), and corresponding to a value 360% over the activity observed in the presence of the Ca^{2+} chelator EGTA. The Ca^{2+} dependent activity was almost completely abolished by the presence in the reaction buffer of the NOS inhibitor L-NMMA. In cells not endowed with the presence of the enzyme (**dox+**), instead, neither L-NMMA nor EGTA caused significant changes in citrulline accumulation, and the absolute values of citrulline formed in the presence of only Ca^{2+} were significantly lower with respect to those obtained in **dox-** cells.

We repeated the same kind of experiment, but instead of lysates using HeLa intact cells that were exposed to ATP, an agonist of (P2Y) receptors known to increase $[\text{Ca}^{2+}]_i$. ATP increased the accumulation of L-citrulline with respect to untreated controls only in eNOS expressing cells (**dox-**); this effect was inhibited by the cell permeant NOS inhibitor L-NAME (see **Figure 2.11B**). We measured $[\text{Ca}^{2+}]_i$ by means of fura-2 fluorimetric analysis in both **dox +** and **dox-** cells after ATP treatment and the results were superimposable, showing a rapid peak increase followed by a return to the baseline (see **Figure 2.17A**)

To investigate whether eNOS expressing cells are able to generate bioavailable NO, we measured formation of cGMP, a good proxy for NO since GC is activated by nanomolar concentration of the gas (**Denninger and Marletta, 1999**). In cells grown in the presence

of dox exposure to ATP for 15 minutes did not result in any increase in cGMP over non stimulated control cells (0.38–0.1 and 0.35–0.1 pmol/mg min⁻¹ respectively, n=4). In eNOS-expressing cells, administration of ATP resulted in a modest increase in cGMP formation over controls (0.44–0.1 and 0.33–0.1 pmol/mg min⁻¹, respectively with n=3) suggesting that the cells are expressing very low levels of sGC.

Taken together the results summarized up to now have revealed limitations of the HeLa cell system to investigate NO generation. Therefore, to assess the formation of bioavailable nitric oxide upon cell treatment, we chose to integrate HeLa with another cell type, the rat pheochromocytoma PC12, as a reporter system: these cells were chosen because they respond to NO with a dramatic increases in cGMP (0.33–0.1 and 9.4–1 pmol/mg min⁻¹ before and after exposure to 100 µM of the NO donor SNAP, respectively, n=3), whereas they do not generate NO when treated with ATP (untreated cells 0.33–0.1 pmol/mg min⁻¹; ATP treated cells 0.35–0.1 pmol/mg min⁻¹).

As shown in **Figure 2.12**, co-incubation of HeLa with PC12 cells caused a rise of cGMP after administration of ATP, which was inhibitable by the preincubation of L-NAME in the cell mixture. As expected, no changes in cGMP content were observed with cells grown in the presence of doxycycline.

The results reported so far indicate that in our eNOS Tet-off system is endowed with a functional eNOS that can be controlled in term of expression. This property offered us a flexible and informative cellular tool to directly challenge the question of whether eNOS expression has consequences in term of regulation of TNF-α triggered apoptosis.

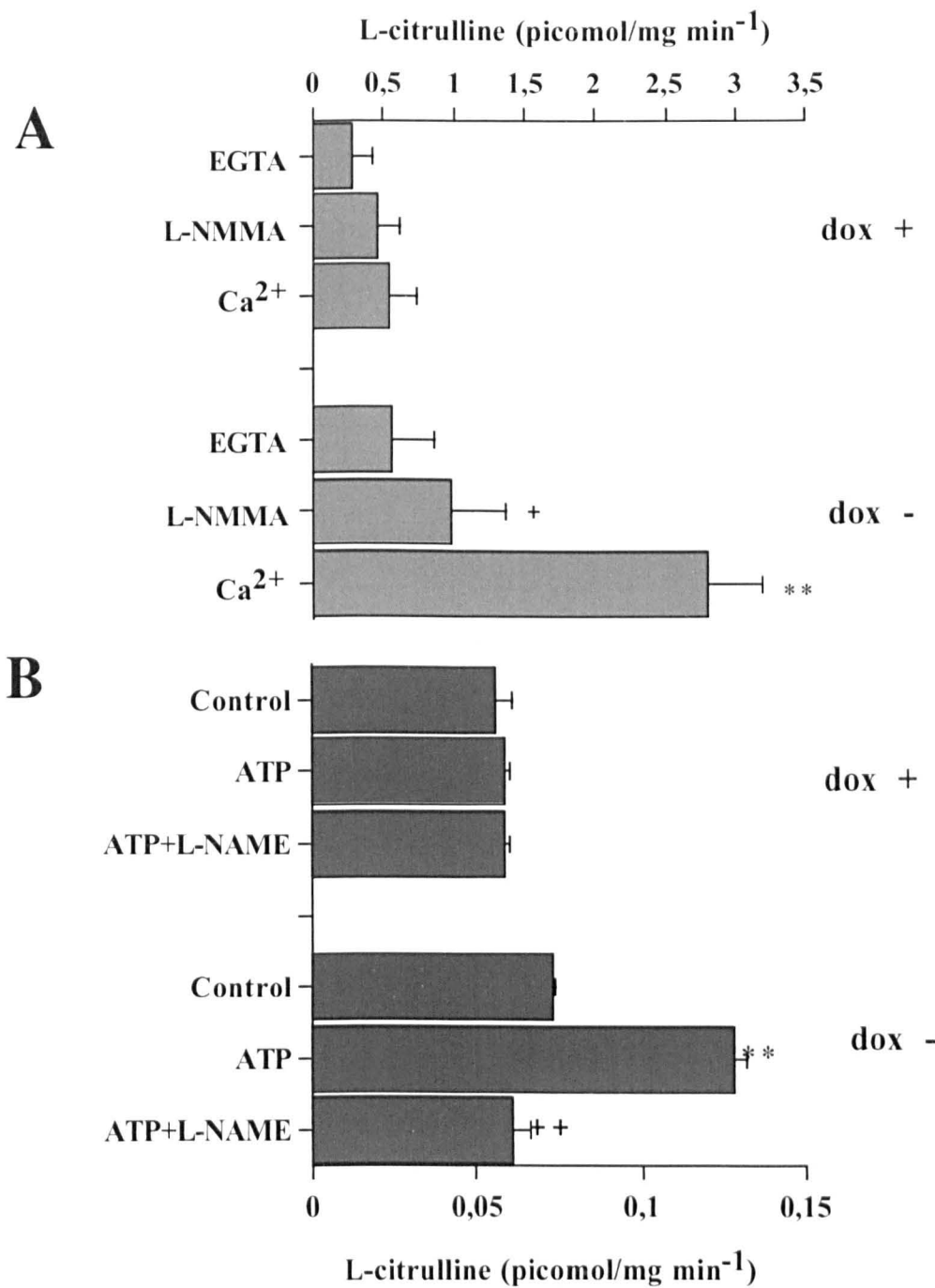


Figure 2.11) Characterization of eNOS activity in HeLa tet-off cells

- A)** Ca²⁺ dependence of eNOS activity in cell lysates prepared from cells grown in the presence (*dox+*) or in the absence (*dox-*) of doxycycline for 72 hours. Lysates had been resuspended in a L-[³H]-Arginine containing reaction buffer containing 0.45 mM CaCl₂ (**Ca²⁺**), 0.45 mM CaCl₂ and 1 mM EGTA (**EGTA**) or 0.45 mM CaCl₂ and 500 μM L-NMMA (**L-NMMA**), and incubated for 10 minutes at 37_i.
- B)** Ca²⁺ dependence of eNOS activity in intact cells. Cells monolayers, grown in the presence or absence of doxycycline (indicated as above) were incubated for 15 minutes at 37_i in a L-[³H]-Arginine medium, alone (**Control**), in the presence of 100 μM ATP (**ATP**) or with 100 mM ATP and 500 μM L-NAME (**ATP +L-NAME**).

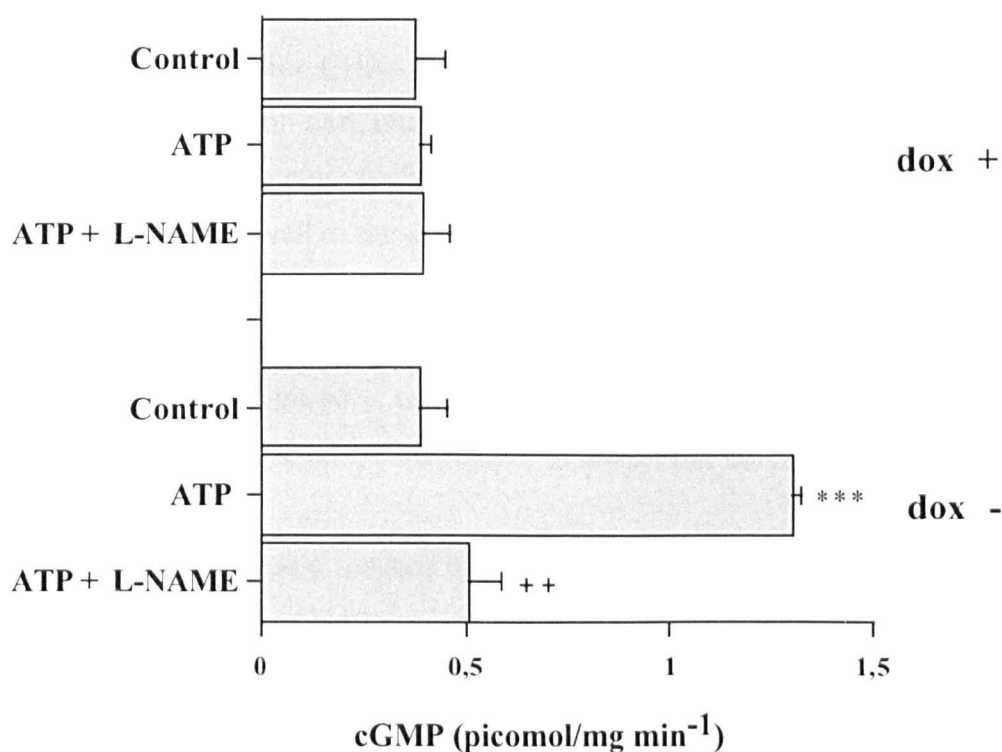


Figure 2.12) cGMP generation by the HeLa/PC12 reporter system

cGMP generation induced in PC12 cells induced by activation of eNOS in HeLa cells. 2×10^6 HeLa tet-off cells, grown in the presence or absence of doxycycline, were resuspended together with 0.5×10^6 PC12 cells and incubated for 15 minutes at 37°C in the same conditions described in the legend of Figure 11B. cGMP accumulation was estimated by a radioimmunoassay and normalized to protein content.

Statistical significance indicated by asterisk is calculated versus untreated control cells; when indicated by the crosses it is instead calculated versus the absence of L-NAME.

2.6) eNOS expression modulates TNF- α induced cell death

To investigate the effect of eNOS expression on TNF- α induced cell death, cells grown in presence or absence of doxycycline were treated overnight with TNF- α together with the protein synthesis inhibitor CHX. To characterize the system I have measured by flow cytometry two different and independent aspects of cell death: the appearance of phosphatidylserine to the outer leaflet of the plasma membrane (as measured by fluorescent Annexin-V staining) as well as the formation of small size hypodiploid DNA fragments.

Results are shown in **Figures 2.13 and 2.14**. Annexin V stained cells were indistinguishable from unstained cells (not shown), and basal levels of Annexin V staining were the same in cell cultured in the presence (**Figure 2.13a**) and in the absence (**Figure 2.13c**) of doxycycline. Therefore expression of eNOS has no effect on basal levels of cell death.

Incubation with TNF α -CHX resulted in both cases in the appearance of annexinV-positive cells, reflecting the increased exposure of phosphatidylserine to the plasma membrane (**Figures 2.13b and d**), albeit at a significantly lower extent in eNOS expressing cells with respect to cells not expressing the enzyme (see also **table 2.2** for the quantification). Similar results were obtained with the analysis of the hypodiploid DNA content. After treatment with TNF- α /CHX levels were significantly higher in eNOS-negative (**Figure 2.14b**) than in eNOS-positive cells (**Figure 2.14d**, see also **Table 2.2** for quantification). These results show that expression of eNOS is sufficient to exert an inhibitory modulation of the TNF- α apoptogenic signalling.

Next question was whether the activity of the enzyme, and hence the production of NO, is necessary to exert this modulatory action. In order to investigate the problem I have repeated the above described experiments in presence of the NOS inhibitor, L-NAME. In untreated cells treatment of dox- cells (bottom row in **Figure 2.13**) cells with this inhibitor did alter neither AnnexinV (**Figure 2.13e**) staining nor the levels of hypodiploid DNA content (**Figure 2.14e**): by contrast when TNF- α /CHX was administered to NOS-positive cells, both parameters were significantly increased to levels that were not significantly different to those observed in the non induced cells.

This effect was specific since in dox+ cells L-NAME preincubation did not have any effect (see **Table 2.2** for quantification). These results confirm that the expression of functional

eNOS is able to exert an inhibitory effect on TNF- α induced apoptosis via the generation of NO.

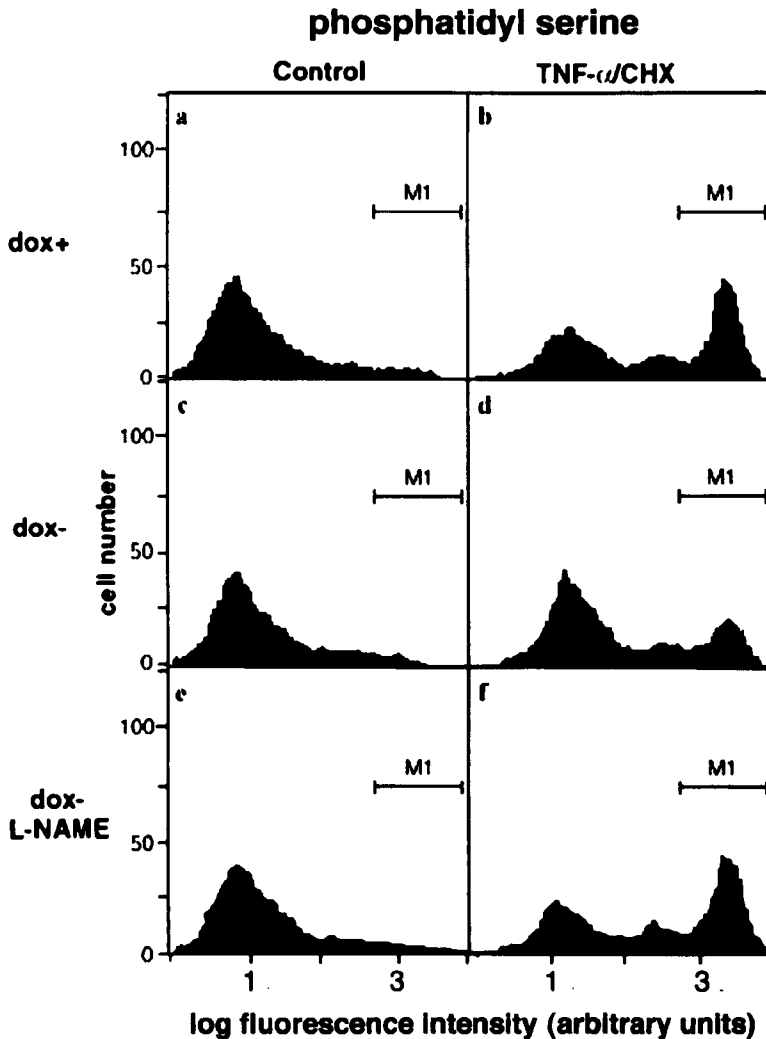


Figure 2.13) Effect of eNOS expression on TNF- α -induced phosphatidyl serine exposure

Cell monolayers, grown in the presence (*dox+*) or in the absence (*dox-*) of doxycycline were incubated for an additional 12 hours without (**Control**) or with (**TNF- α /CHX**) TNF- α (100 ng/ml) plus CHX (1 μ g/ml). L-NAME (500 μ M) was added to the incubation where indicated. Annexin V staining was measured with the cytofluorimeter. No significant AxV staining was seen in control cells exposed only to the protein synthesis inhibitor (for *dox+* percentage of cells present in the data region considered for statistical analysis in the presence of only CHX was 5–1%, for *dox-* cells it was 6–2)

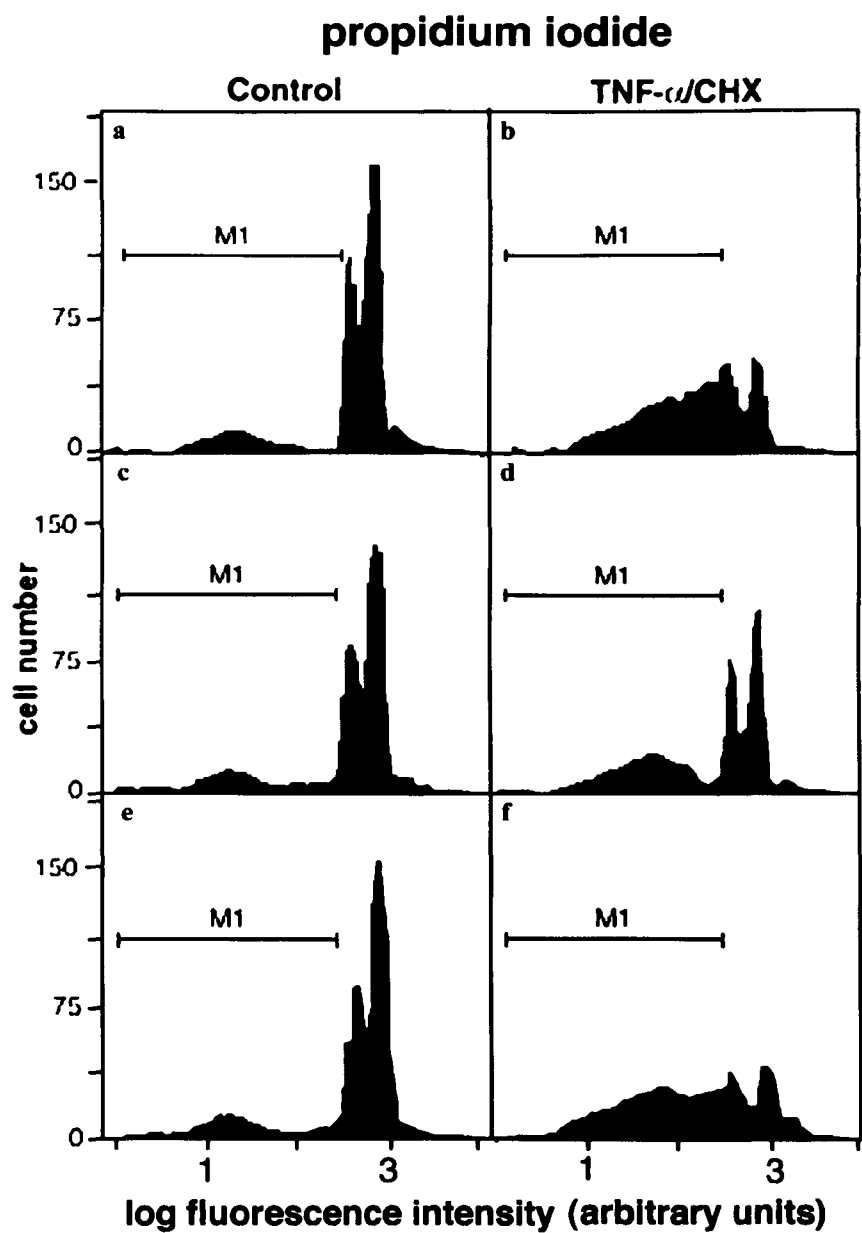


Figure 2.14) Effect of eNOS expression on TNF- α induced hypodiploid DNA increase

Cells grown and incubated as in the **Figure 2.13** legend, were fixed. Propidium iodide staining of the fixed cells was assayed to measure DNA content. In these experiments HeLa cells were not synchronized. Accordingly G0/G1, S and G2/M phases were present as already described for HeLa cells (see **Rodriguez and Flemington, 1999**). The sub-G1, hypodiploid peak, characteristic of apoptosis is clearly detectable in *b*, *d* and *f* with respect to the control cells (*a*, *c* and *e*). M1 indicates the region considered for statistical analysis. The pre-treatment of cells with CHX only did not alter the number of cells with an hipodiploid DNA content in the region considered for statistical analysis: dox+ cells 17–4%, dox- cells 15–5%.

Table 2.2: effects of eNOS expression on the TNF- α -induced exposure of phosphatidyl serine and formation of hypodiploid DNA

		annexin V staining		propidium iodide staining	
		control	TNF- α /CHX	control	TNF- α /CHX
dox +	5-1	41-2	*** +++	17-1	*** ++
dox+ / L-NAME	5-1	45-2	***	18-1	**
dox -	5 - 1	21-1	***	16-1	***
dox- / L-NAME	7-1	43-2	*** +++	17-1	*** ++

HeLa A δ cells, cultured in the presence (dox+) or absence (dox-) of doxycycline, were either treated with TNF- α (100 ng/ml) and CHX (1 M) for 12 h, or left untreated (control), before flow cytometry analysis of annexin V and propidium iodide staining, as described under Materials and Methods chapter. Values represent the percentage of cells measured in the M1 regions, defined as shown in Fig. 2.13 and 2.14. Statistical probability vs controls is indicated by the asterisks, that of dox+ vs. dox- and dox-/L-NAME vs. dox- cells after TNF- α /CHX treatment by the crosses, both calculated as described under Materials and Methods ($n = 4$).

2.7) TNF- α stimulation increases eNOS activity and NO generation

To investigate whether the effect of NO modulation was exerted by a tonic activation of eNOS or by an acute stimulation of the enzyme following apoptotic stimuli, we investigated the effect of a 15 minute stimulation with the cytokine on the arginine/citrulline assay in intact cells, as well as the production of cGMP in the HeLa/PC12 reporter system.

As shown in **Figure 2.15A**, citrulline accumulation was stimulated by TNF- α in eNOS expressing HeLa cells (dox-). Consistently, co-incubation of HeLa cells stimulated with TNF- α with PC-12 induced an increased cGMP generation in the latter (**Figure 2.15B**). Both of these effects were prevented by the preincubation of HeLa cells with the NOS inhibitor, L-NAME. Of importance, neither of these effects was observed in cells not expressing eNOS (dox+). I next investigated the pathway through which TNF- α induces eNOS activation. The cytokine did not induce any increases in $[Ca^{2+}]_i$, ruling out a major role of the cation in eNOS activation (**Figure 2.15C**). I then studied whether TNF- α induced eNOS activation is due to stimulation of the Pi3K/Akt pathway, with ensuing activation of eNOS phosphorylation in the Ser1179 residue. To this end, I carried out Western blot experiments with an antibody recognizing eNOS specifically phosphorylated in Ser1179, standardized by using a monoclonal antibody recognizing both the phosphorylated and the unphosphorylated form of the enzyme. These blots revealed the appearance of a phospho-specific band in the cells stimulated with 10 minutes TNF- α (**Figure 2.15D**).

As shown in **Fig 2.16A**, TNF- α treatment of HeLa cells resulted also in the phosphorylation of Akt on its Ser 473 residue (associated with activation, see **Alessi et al, 1996**), which was specifically inhibited by preincubation of the cells with the Pi3K inhibitor, wortmannin. Remarkably the wortmannin preincubation also inhibited eNOS activation, as showed by western blot (see **Figure 2.16B**) as well as by arginine/citrulline assay (see **Figure 2.16C**).

These results indicate that eNOS activation by TNF- α occurs through activation of the Pi3K/Akt pathway and phosphorylation of eNOS on its Ser 1179 residue.

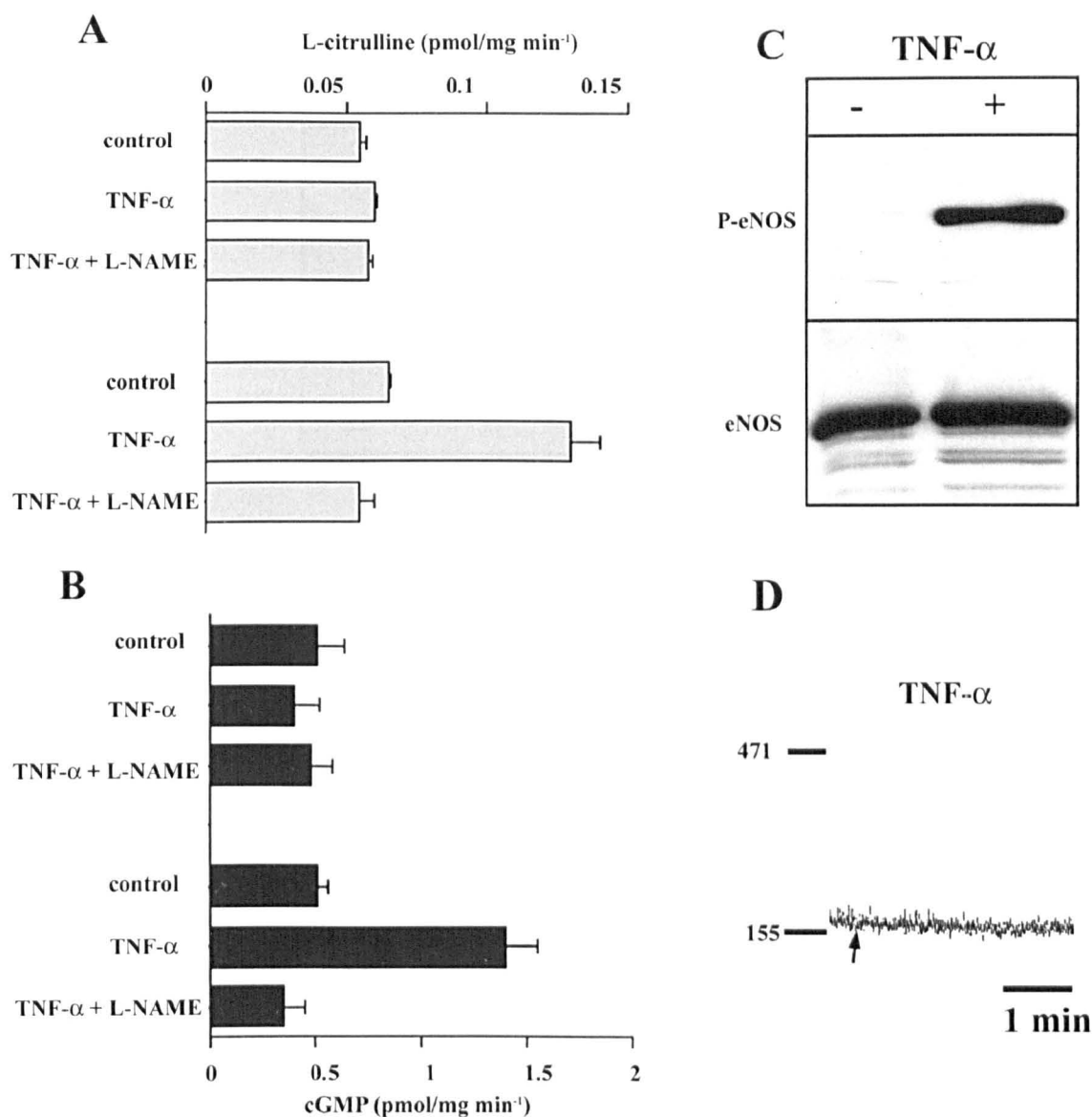


Figure 2.15) Effect of TNF- α treatment on NO generation in HeLa/tet-off cells

- A)** eNOS activity induced by TNF- α assayed in intact cells. Cell monolayers grown with (*dox*+) or without (*dox*-) doxycycline, were incubated for 15 minutes at 37°C in a medium containing L-[³H]-arginine with or without TNF- α (100 ng/ml) and/or L-NAME (0.5 mM)
- B)** effects of TNF- α treatment on cGMP generation by the PC12 reporter system. 2×10^6 HeLa cells were resuspended with 0.5×10^6 PC12 cells, and were treated for 15 minutes without (*control*) or with (*TNF- α*) TNF- α (100 ng/ml) and without or with L-NAME (0.5 mM; *TNF- α + L-NAME*)
- C)** Western Blot analysis of HeLa *dox*- cells treated for 10 minutes with TNF- α (100 ng/ml). The upper panel: antibody against phosphorylated eNOS. Lower panel: same membrane immunoblotted with an antibody against total form of eNOS.
- D)** [Ca^{2+}]_i levels induced by TNF- α in eNOS tet-off cells loaded with the Ca^{2+} sensitive fluorescent dye fura-2AM. The stimulus was added when indicated by the arrows, and the [Ca^{2+}]_i scale is reported on the left hand side.

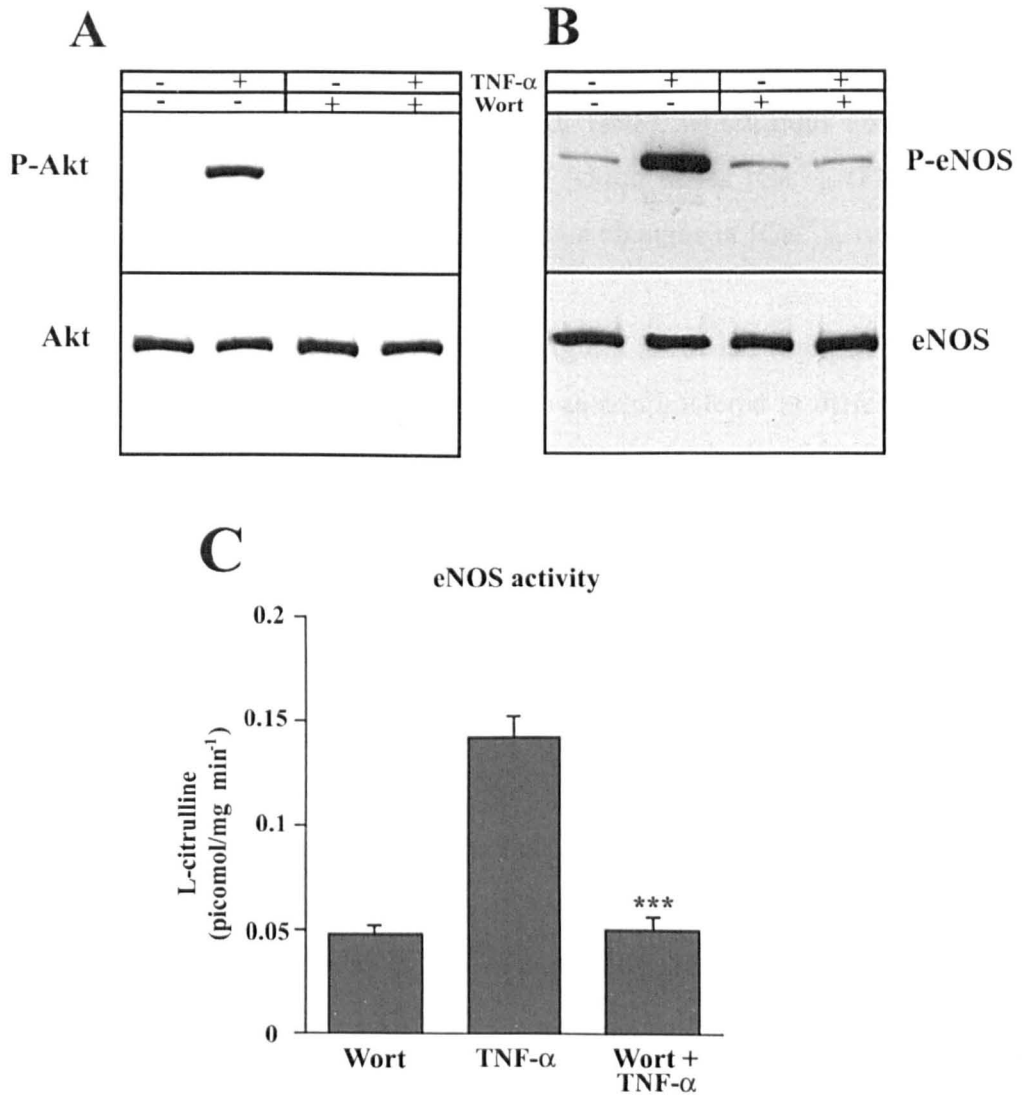


Figure 2.16) eNOS activation by TNF- α involves Pi3K activation.

eNOS expressing HeLa cells were treated for 10 minutes with TNF- α (100 ng/ml), with or without wortmannin (Wort, 100 nM). Cells were lysed and samples analysed by western blot with antibodies recognizing (A) either the phosphorylated Ser473 form (upper panel; *P-Akt*) or the whole Akt (lower panel: *Akt*). Western blot analysis was also performed with antibodies recognising eNOS (B), either in its phosphorylated Ser 1179 form (upper panel; *P-eNOS*) or as a whole (lower panel; *eNOS*).

C) I have also measured eNOS activity in the same conditions as above, measuring the arginine/citrulline conversion by whole cells. Statistical probability is indicated by the asterisks and calculated vs cells treated with only TNF- α . (n=5)

TNF- α induced activation of eNOS had not been described previously, so I decided to characterize further this pathway by comparing the time course of activation of the enzyme by the cytokine with that induced by other two stimuli: EGF, a growth factor known to directly activate the Pi3K/AKT signalling (**Wang et al, 1998**), which does not increase $[Ca^{2+}]_i$ in our cells (**Fig 2.17A**), and ATP, an agonist which raises $[Ca^{2+}]_i$ (**Fig 2.17A**) without concomitant Akt activation. TNF- α did not induce changes in $[Ca^{2+}]_i$, ruling out a possible involvement of the cation in the pathway.

In order to carry out these experiments, I adopted a slightly modified arginine/citrulline assay on whole cells, where the radioactive substrate was administered at different time-points after the challenge with the stimuli. This experimental protocol gave us the opportunity to measure the time-course of L-arginine conversion to L-citrulline rather than the accumulation of the latter as evaluated in the previous experiments.

Results of this approach are shown in **Figure 2.17B**. The percentage value reported for each time point represents the value of citrulline accumulation obtained with the indicated stimuli versus the citrulline accumulated in untreated cells, run in parallel as internal controls.

ATP caused a transient citrulline accumulation which peaked at 2 minutes and rapidly faded out, returning to the basal levels after 6 minutes: this profile of activation is consistent with the rapid and transient peak of $[Ca^{2+}]_i$ caused by the compound and showed by fluorimetric fura-2 analysis in **Figure 2.17A**.

In contrast to ATP, TNF- α gave rise to a slower activation of the enzyme (see **Figure 2.17B**). Citrulline levels began to rise with an appreciable delay from cytokine administration, being visible after 6 minutes, with a peak after 10 minutes and return to the baseline after 15 minutes. In contrast, EGF treatment resulted in a rapid onset of citrulline accumulation, with levels remaining above controls until 20 minutes and then declining to resting levels within half an hour from the start of the treatment. None of these effects were present in cells not endowed with eNOS expression (data not shown).

I then compared the time course of activation of eNOS, measured by citrulline accumulation, with the activation of Pi3K/AKT pathway and eNOS phosphorylation. The patterns induced by TNF- α and EGF were quite different. EGF stimuli did cause a rapid activation of AKT, already visible after two minutes of treatment with the growth factor, while treatment with TNF- α followed the same delay as observed by measuring eNOS activity, with a strong signal visible after 5 minutes from the treatment with the cytokine.

EGF induced phosphorylation of eNOS with a time course matching closely that of eNOS activity, with a rapid increase followed by a decrease after 20 minutes, and by a complete switch off after 30 minutes.

Similarly, the pattern of TNF- α -induced eNOS phosphorylation matched that observed by measuring eNOS activation. TNF- α phosphorylated eNOS with a delay comparable to that induced by EGF, reaching a peak after 10 minutes, decreasing at 15 minutes and reaching basal levels after 20-30 minutes from initial stimulation. Of importance, Akt phosphorylation induced by TNF- α persisted longer (half an hour, with complete switch-off after 1 hour: data not shown) compared to eNOS phosphorylation and activity, whereas a dissociation between Akt and eNOS was not observed with EGF. ATP caused neither Akt nor eNOS phosphorylation.

Taken together these results show that the Pi3K/Akt module signalling pathway, although able to sustain and regulate the activation of eNOS by TNF- α and EGF, is differentially regulated.

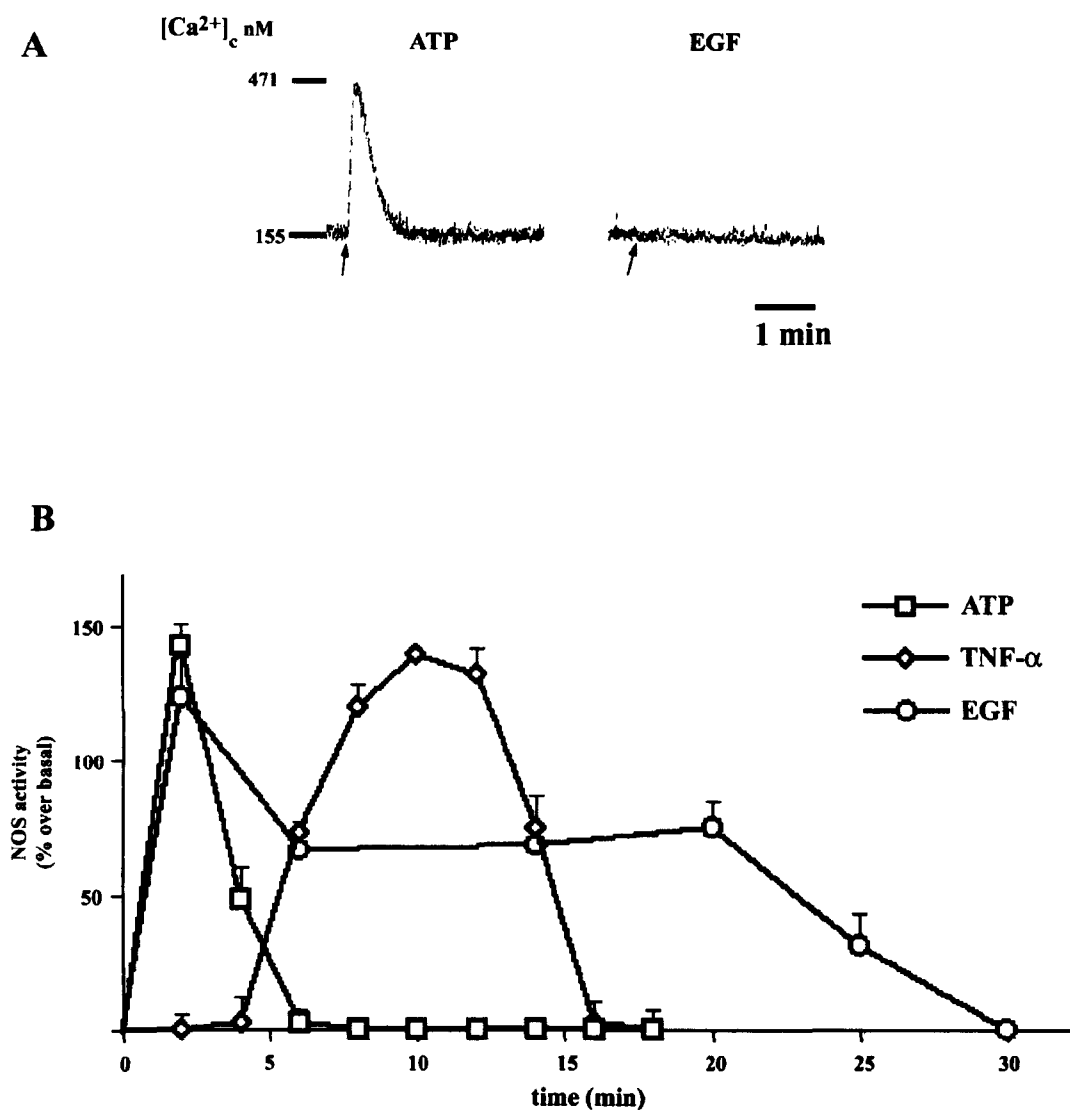


Figure 2.17) Different pathways of eNOS activation.

- A)** $[Ca^{2+}]_i$ increases induced by ATP and EGF in eNOS tet-off cells loaded with the Ca^{2+} sensitive fluorescent dye fura-2AM, estimated at the cytofluorimeter. The different stimuli were added when indicated by the arrows, and the $[Ca^{2+}]_i$ scale are reported on the left hand side. Traces are representative of 5 experiments
- B)** eNOS-expressing HeLa tet-off cells were treated with ATP (100 μ M), EGF (100 ng/ml) or TNF- α (100 ng/ml) at time 0. L- $[^3H]$ -arginine was added at the time points indicated in the X axis; the reactions were stopped every 2 minutes. eNOS activity was estimated as pmol/min of L- $[^3H]$ -citrulline formed in the 2 minutes time window, and normalised to the protein content. Results are here expressed as mean % of the increased L- $[^3H]$ citrulline formation over basal, i.e. the untreated cells.

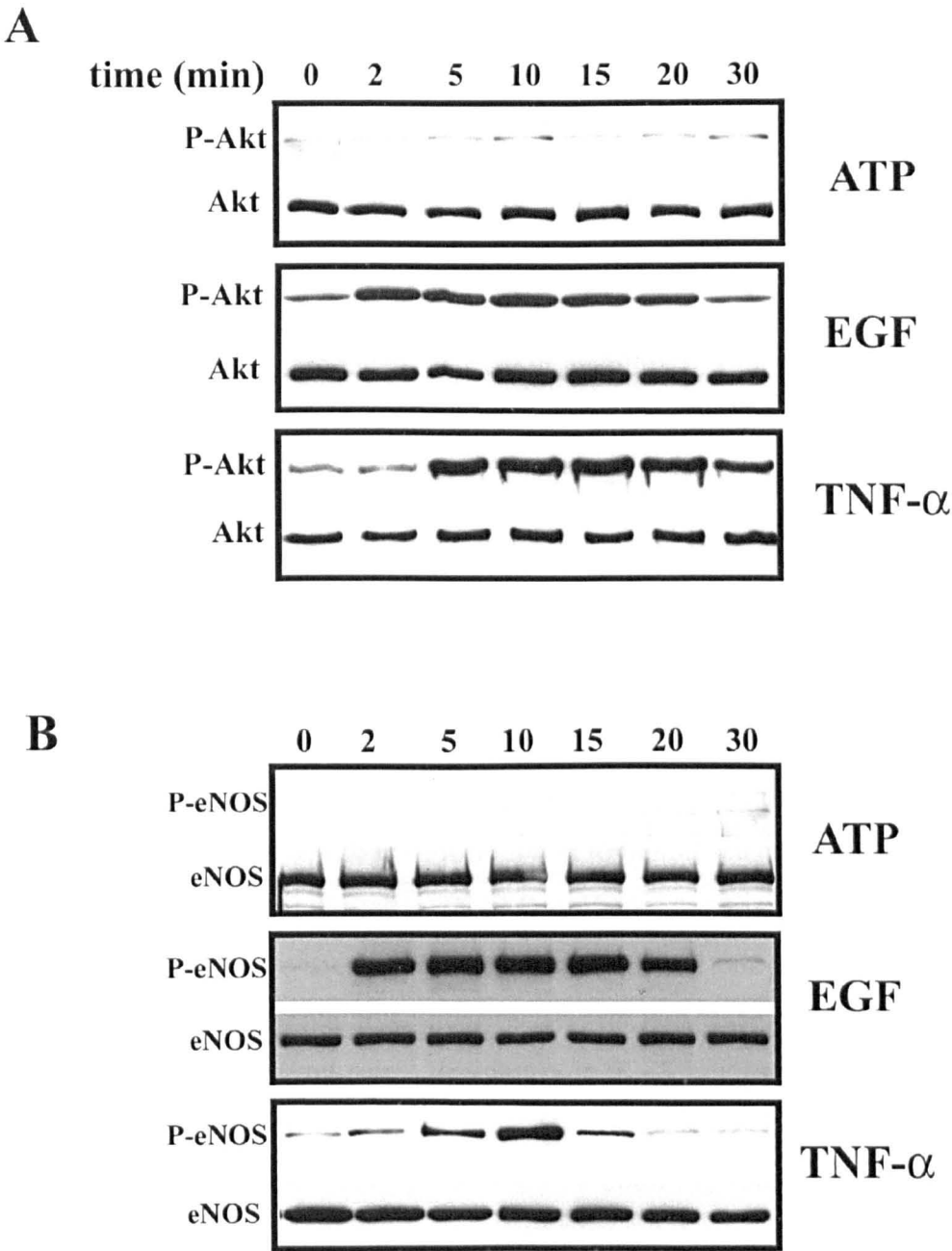


Figure 2.18) Patterns of Akt (**A**) and eNOS (**B**) phosphorylation by different stimuli

eNOS expressing HeLa tet-off cells were treated with ATP (100 μ M), EGF (100 ng/ml) and TNF- α (100 ng/ml) and the reactions stopped at the indicated time points, by quick transfer of the dishes to ice and lysing of the cells. Western blot of active, phosphorylated Akt (**A**: *P-Akt*) and eNOS (**B**; *P-eNOS*) vs total (*Akt* and *eNOS*), non-phosphorylated enzymes was estimated out as indicated in Figure 2.16 legend.

2.9) Role of sphingomyelinases in TNF- α -induced activation of eNOS

eNOS had been previously reported to be activated by exogenous ceramide (**Igarashi et al, 1999**): I thus decided to investigate the role of SMase activities in the context of TNF- α triggered eNOS activation. eNOS-Tet off cells were treated with TNF- α for 5 minutes and SMase activity was measured in cell lysates at the optimum pH for either enzyme (pH 5.5 and 7.4 for A-SMase and N-SMase, respectively), using the same protocol already described for U937 cell line. Briefly, SMase activity was evaluated as the increase of radioactive phosphorylcholine produced after the stimulation of cell aliquots with TNF- α . As shown in **Figure 2.19A and B**, both SMase are activated upon the cytokine stimulation, with phosphorylcholine accumulation that was incremented by 105% over the basal in the case of N-SMase while it was of around 60% in the case of A-SMase (see **figure 2.19** legend for the absolute values).

I have then employed a panel of inhibitors, namely imipramine and D609 for A-SMase and scyphostatin and manumycin A for N-SMase, as suggested by previous reports in the literature (**Jensen et al, 1999; Cifone et al, 1995; Tanaka et al, 1997; Arenz et al, 2001** respectively) and by our results U937 cells (**Fig 2.19 A and B**). At the concentration used D609 (25 μ g/ml) and imipramine (20 μ M) were found to inhibit selectively TNF- α induced A-SMase activation, while scyphostatin (1 μ M) and manumycin A (5 μ M) appeared specific for the N-SMase activity. When administered alone none of the drugs had any effect on the basal level of sphingomyelin hydrolysis (for **A-SMase**: D609 31–5 pmol/mg min⁻¹; imipramine 29–6 pmol/mg min⁻¹; scyphostatin 32–5 pmol/mg min⁻¹; manumycin 33–5 pmol/mg min⁻¹. Regarding **N-SMase** values were: D609 10–4 pmol/mg min⁻¹; imipramine 11–5 pmol/mg min⁻¹; scyphostatin 9–4 pmol/mg min⁻¹; manumycin 10–5 pmol/mg min⁻¹).

The same panel of inhibitors was also tested by western blot analysis (**Figure 2.20**) and by whole cell eNOS assay (**Figure 2.21**) to try to pharmacologically dissect the contribution of the various SMase activities to AKT and eNOS activation. As shown in **Figure 2.20** treatment for 5 minutes with TNF- α resulted in the activation of both Akt (upper panels) and eNOS (lower panels). The use of N-SMase inhibitors did completely abolished the TNF- α triggered phosphorylation of both enzymes, while the A-SMase inhibitors did not have any effect.

Consistent with these results, I found that, similar to the results obtained above, L-citrulline induced by TNF- α , was prevented by co-incubation with scyphostatin or manumycin while D609 and imipramine had no effect. These results strongly suggest that in this system N-SMase mediates eNOS activation while A-SMase seems not involved in the process (**Fig 2.21**).

Wortmannin preincubation did change neither the basal rate of sphingomyelin conversion to phosphorylcholine nor the increase in phosphorylcholine accumulation induced by TNF- α , indicating a site of action downstream of N-SMase activation (**Fig 2.22**).

Taken as a whole, these results indicate that TNF- α activates eNOS through a novel signaling pathway involving the sequential activation of N-SMase and Pi3K/Akt. This pathway is different from that activated by EGF, whose kinetic has been shown in figure19B, which occurs through activation of Akt without involvement of SMases.

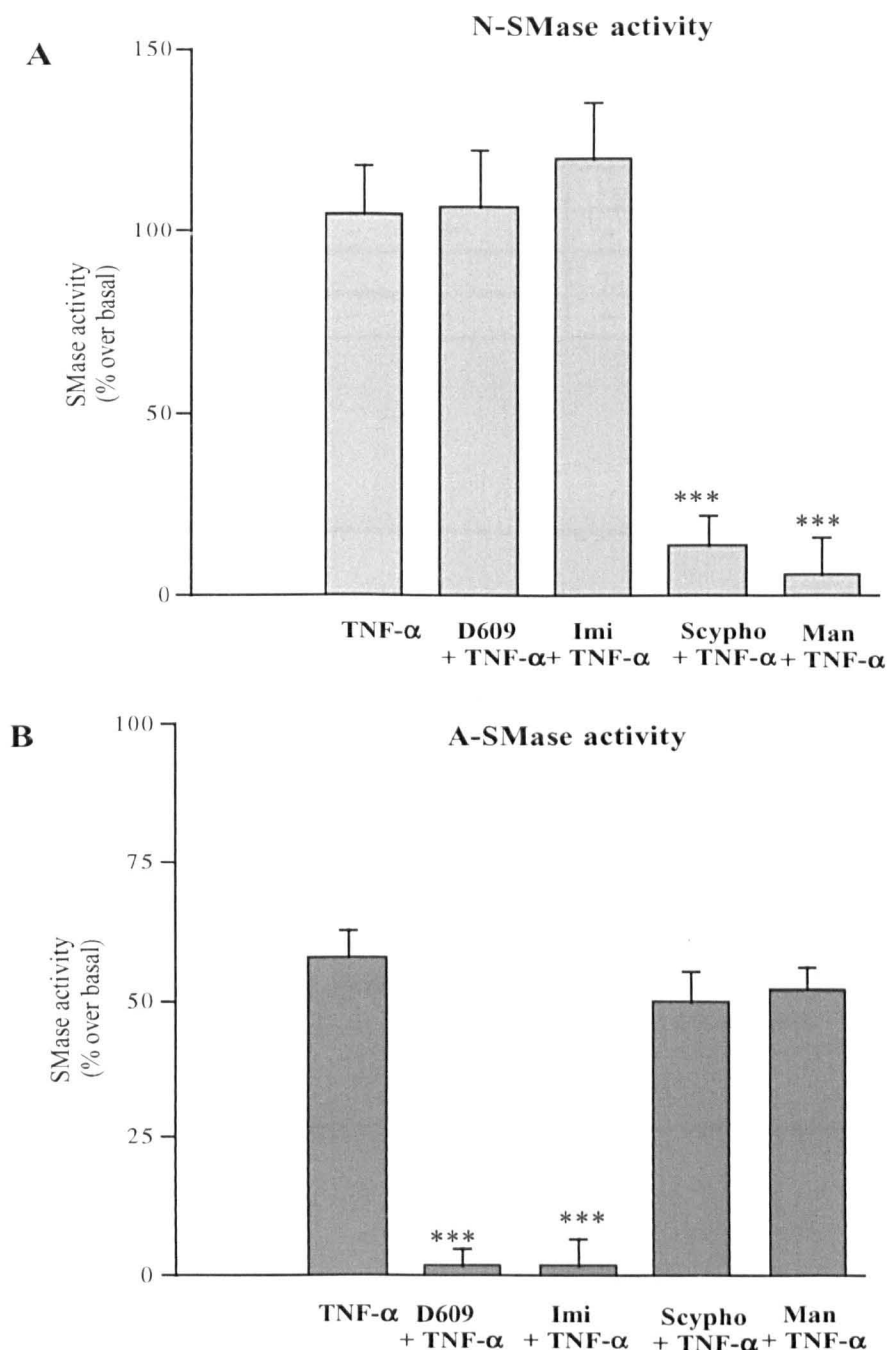


Figure 2.19) TNF- α activation of SMases in HeLa tet-off cells: specificity of SMase inhibitors.

eNOS expressing tet-off cells were treated for 5 minutes with TNF- α (TNF- α ; 100 ng/ml), with or without either D609 (D609: 25 μ g/ml), imipramine (Imi: 20 μ M), scyphostatin (Scypho: 1 μ M) or manumycin A (Man: 5 μ M) as indicated. The two histogram graphs shows the specificity of the action of the various SMase inhibitors on the TNF- α triggered N-SMase (A) or A-SMase (B) activities, determined in cell lysates by measuring [N-methyl- 14 C]-sphingomyelin hydrolysis to phosphorylcholine at either pH 7.4 (A: N-SMase activity) or 5.5 (B: for A-SMase activity). Values were expressed as % – s.e.m. over the SMase activity measured in untreated cells (10–4 pmol/mg min $^{-1}$ and 33–6 pmol/mg min $^{-1}$ for N-SMase and A-SMase respectively) (n=5)

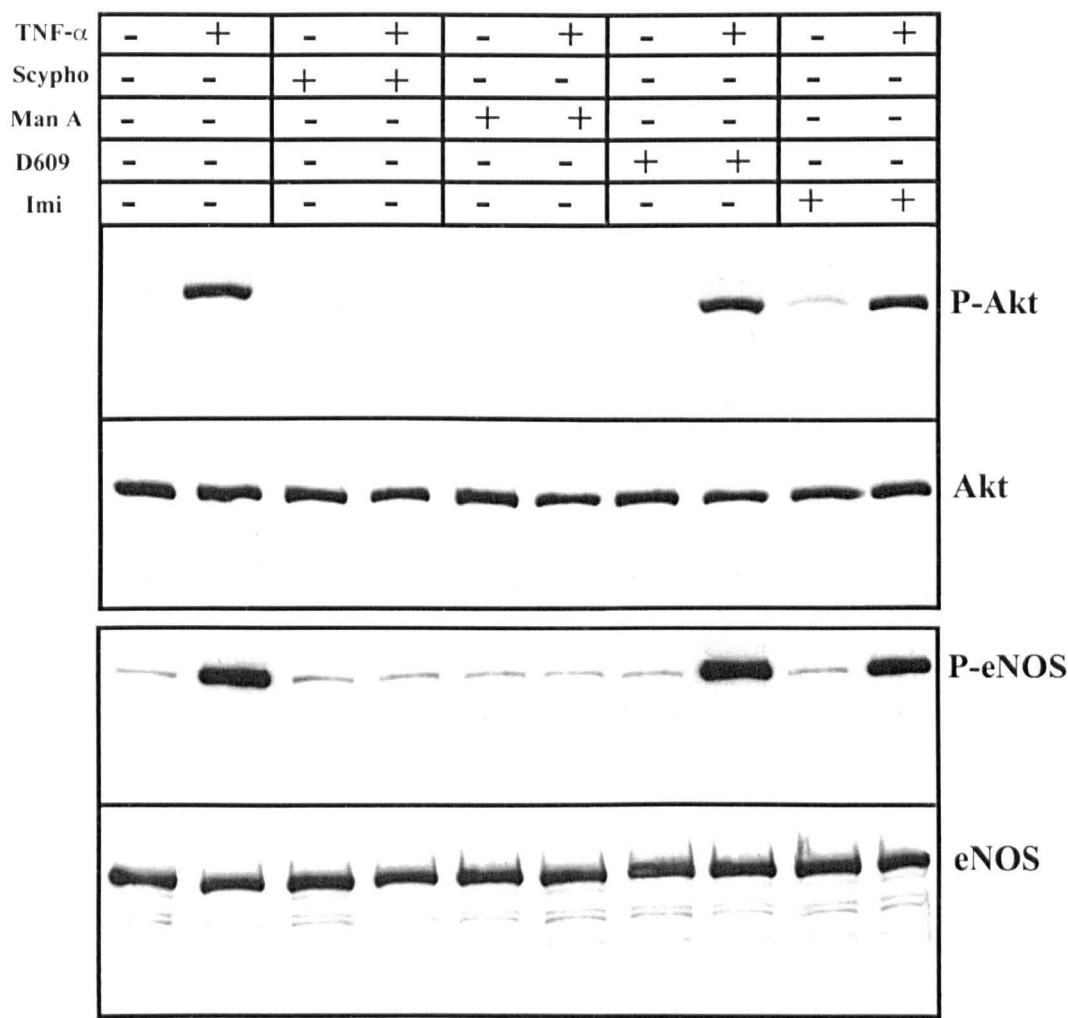


Figure 2.20) TNF- α induced phosphorylation of Akt and eNOS takes place downstream to N-SMase: Western Blot analysis.

eNOS expressing HeLa tet-off cells were treated for 10 minutes with TNF- α (100 ng/ml) with or without scyphostatin (*Scypho*: 1 μ M), manumycin A (*Man A*: 5 μ M), D609 (*D609*: 25 μ g/ml) or imipramine (*Imi*: 20 μ M), as indicated. Analysis of phosphorylated, active Akt (P-Akt) and eNOS (P-eNOS) was performed by western blots, carried out as described in the legend of **Figure 2.16** (n=4)

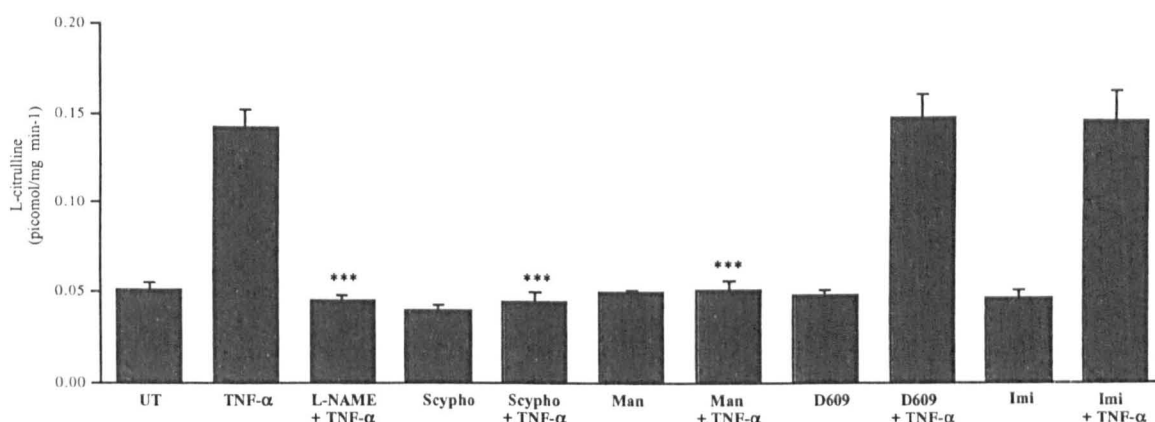


Figure 2.21) TNF- α induced activation of eNOS takes place downstream to N-SMase: arginine/citrulline assay

eNOS expressing HeLa tet-off cells were left untreated (*UT*) or treated for 10 min with TNF- α (100 ng/ml), with or without L-NAME (0.5 mM). Incubations with SMase inhibitors were carried on as indicated in **Figure 2.19**. eNOS assay was carried out in whole cell preparations, as already described, and activity of Arginine/Citrulline conversion was estimated as pmol/min of L-[3H]citrulline formed in the reaction. Results are expressed as means – s.e.m. ($n=5$); statistical significance is indicated by the asterisks and calculated versus cells treated only with TNF- α .

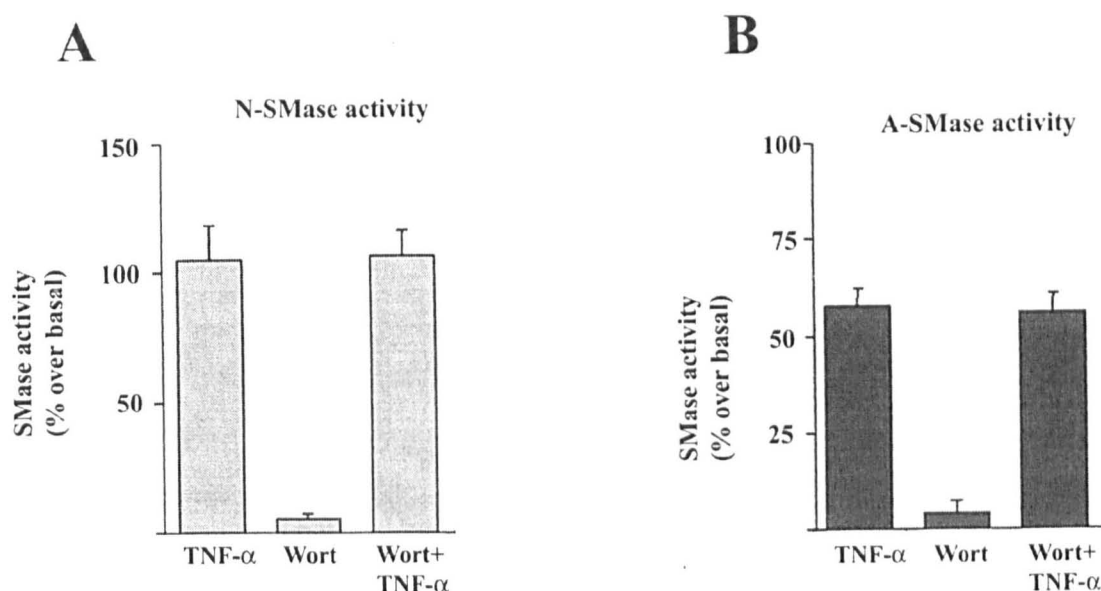


Figure 2.22) TNF- α induced SMase activation is Pi3K independent

eNOS-expressing tet-off cells were treated for 5 minutes with TNF- α (100 ng/ml), with or without wortmannin (Wort, 100 nM) as indicated. Analyses of **(A)** N-SMase (absolute value in untreated cells: $10\text{--}4\text{ pmol/mg min}^{-1}$) and **(B)** A-SMase (absolute value in untreated cells: $33\text{--}6\text{ pmol/mg min}^{-1}$) activation ($n=3$) were carried out and reported as described in the legend of **Figure 2.19**. Statistical significance is indicated by the asterisks and calculated vs cells treated with only TNF- α .

Chapter 3

DISCUSSION

The present thesis includes two distinct, yet coordinate, set of studies. The first set was aimed at analyzing the role of NO released from an exogenous chemical source on proximal events of TNF- α apoptotic signal transduction, investigated in the well characterized monocytic cell line U937. In previous studies the U937 line had been widely used to investigate TNF- α signalling. The second set concerned the role of endogenously generated NO on the same TNF- α apoptotic signalling. For these studies I used a different cellular model, generated for this specific purpose: a HeLa tet-off cell line conditionally expressing eNOS. The utilization of the latter system has allowed the identification of a novel signalling pathway involving the sequential activation of SMase, Pi3K and eNOS.

From here on, I will therefore discuss the two sets of data separately, while in the last and conclusive section I will present a hypothetical model that could help illustrating and rationalizing the complicated signalling network activated by TNF- α . Such model constitutes a working hypothesis to further understand the complex regulation of the cell death process triggered by the cytokine.

3.1) Exogenous NO and TNF- α Signalling

Ceramide has been recently recognised as an important intracellular second messenger, contributing to a wide series of biological processes such as inflammation, cell growth and differentiation. So far, its role has been mainly linked to the induction of apoptotic responses (**Huwiler et al, 2000**). However the direct target of ceramide in induction of apoptosis, as well as its possible role in the regulation of other death inducing stimuli, was not yet established.

Previous results obtained in our lab showed that treatment of U937 cells with ceramide potentiates early TNF- α apoptotic signalling as detected by increases in TRADD recruitment and caspase-8 activation (see **Figures 1.15** and **DeNadai et al, 2000**). Moreover inhibition of endogenous ceramide generation by NO/cGMP was able to exert

inhibitory effects on TNF- α triggered apoptosis. However the targets of the NO/cGMP action on ceramide generation remained undefined..

I thereby decided to focus on SMases as the possible target. This decision was made for two different reasons: the first is that, despite the existence of a de novo ceramide bio-synthetic pathway (described in the Introduction), sphingomyelin appears to be the primary source of ceramide in the cells, emphasizing the role of SMase for its production. The second reason is that, among the various enzyme regulating ceramide concentration in the cell, SMase are activated by TNF- α early enough (**Wiegmann et al, 1994; Schutze et al, 2000**) to account for the initial wave of ceramide generation, potentially responsible for the effects that we had previously shown (**DeNadai et al, 2000**).

I have focused on A-SMase and N-SMase because these were the activities already indicated to contribute substantially to death signalling (**Higuchi et al, 1996; Bezombes et al, 1998; Lozano et al, 2001, Segui et al; 2001; Lin et al, 2000**). Death receptor-triggered mechanism of SMase activation had been studied: both enzymes, A- and N-SMase, are activated early after death receptor stimulation, downstream to recruitment to the cytoplasmic site of receptor for proteins such as FAN for N-SMase (**Adam-Klages et al, 1996; Segui et al, 2001**) and FADD for A-SMase (**Chinnayan et al, 1996; Schwander et al, 1998**). Of importance expression of dominant negative of both of these adaptor protein bring to long lasting inhibition of apoptosis, which was releaved only by administration of exogenous ceramide (**Lozano et al, 2001; Segui et al, 2001**).

To investigate the physiological relevance of SMases in early phases of apoptosis induced by TNF- α I have investigated in our cell model the role of each enzyme in the regulation of caspase 8 activation and in DNA fragmentation. These studies were carried out using a panel of structurally unrelated inhibitors of SMase activities, characterized for their specificity of action on either enzyme. I have found that A-SMase is involved in the regulation of early phases of apoptosis, a result in agreement with a previous report in these cells (**Schutze et al, 1999**). In contrast N-SMase was not involved. The latter enzyme, however, is activated not only at early time points. Infact it undergoes also a second wave of activation, initiated around 90 minutes after treatment with TNF- α . Therefore a late role of N-SMase in the apoptotic process cannot, therefore, be excluded (**Segui et al, 2001; Luberto et al, 2002**).

Current knowledge regarding possible modulation of SMases once they have been activated is very limited. A priori, however, such a regulation might be an important keypoint to the

modulation of the entire pathway. With the present work I have produced evidence that NO might be one of these regulators. In fact NO was able to inhibit in a concentration dependent manner, the TNF- α triggered activation of SMase, beginning in the first minutes after cytokine administration to the cells. This effect was shown to be cGMP dependent as it was mimicked by 8Br-cGMP and inhibited by ODQ. In addition it was found to depend upon activation of PK G, a known cellular target for cGMP. It remains to be established whether this kinase phosphorylates directly SMases or acts through other intermediate protein, as proposed for protein kinase C regulation of N-SMase (**Mansat et al, 1997**).

Of importance in terms of apoptosis is that prevention, observed after inhibition of the A-SMase with the three different blockers, was mimicked by 8Br-cGMP and not increased further by the co-incubation of each of the three inhibitors. This indicates that inhibition of A-SMase by NO/cGMP account for its initial protective effect. This is further confirmed by the observation that both A-SMase- and cGMP-dependent protections operate only during the initial phases of apoptosis, since 8-BrcGMP and A-SMase inhibitors lost their protective effect as apoptosis proceeded, becoming completely ineffective at later stages (6 hours). An important implication of these experiments is that NO/cGMP seems to delay rather than completely inhibit apoptosis, at least in the particular cellular system investigated.

In our cells NO was found to modulate apoptosis through both cGMP-dependent and -independent events. The late stage protection was found to be cGMP-independent and to depend on the oxidative state of the cells, as suggested by its inhibition by the reducing agent DTT (**DeNadai et al, 2000**). Late S-nitrosylation might be indeed facilitated by the fall in cell reducing potential that occurs in the course of TNF- α induced apoptosis (**Higuchi et al, 1998**). GSH is an important inhibitor of N-SMase. Decrease of GSH concentrations during TNF- α triggered apoptosis might provide an explanation for the delayed and persistent activation of N-SMase observed after 60-90 minutes from cytokine addition. The functional role of this late activation has not yet been specifically addressed and could represent a likely and interesting continuation of the present work in the future.

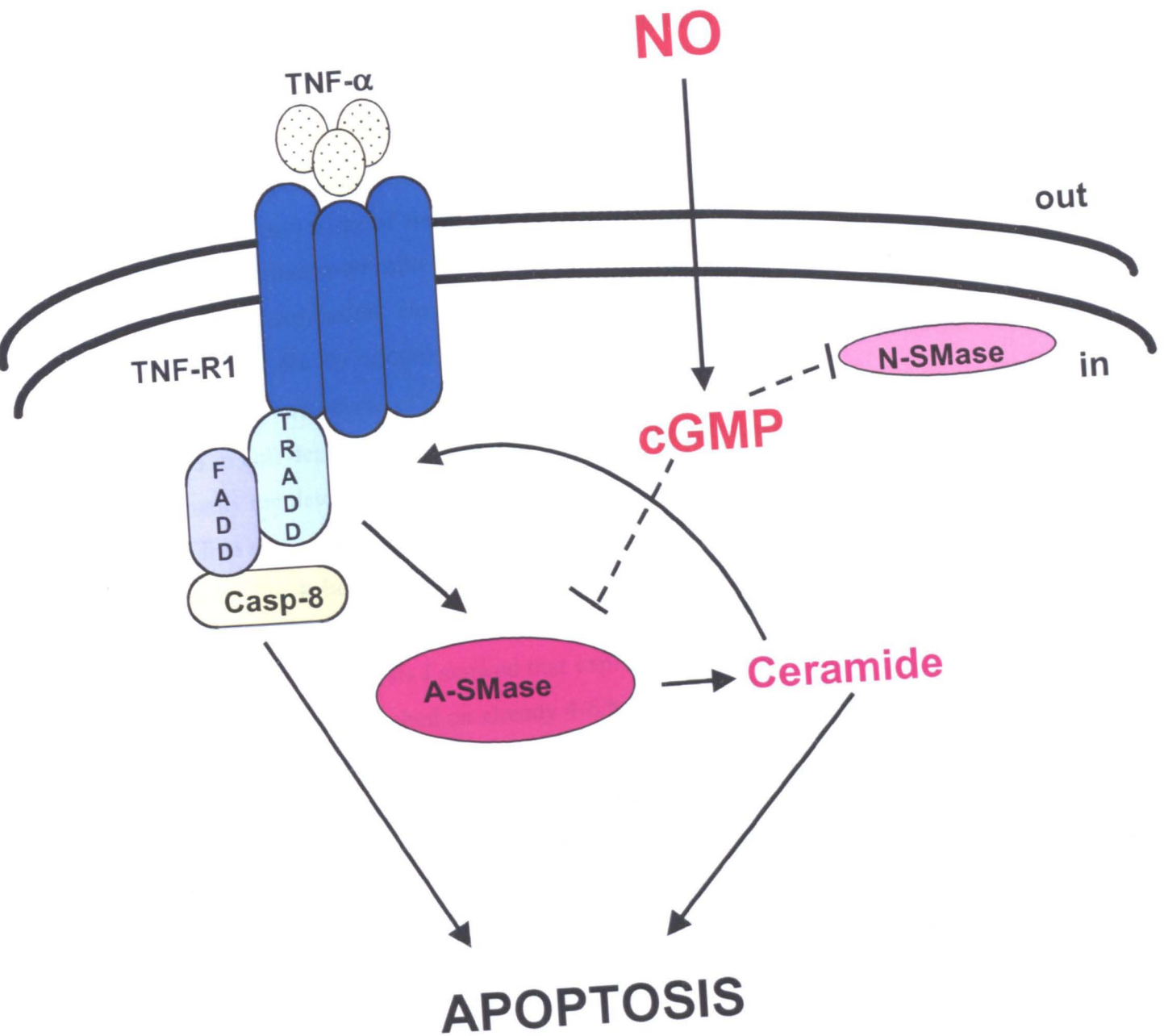


Figure 3.1) NO/cGMP dependent inhibition of SMases and of TNF- α triggered early apoptotic signalling

3.2) Endogenous NO and TNF- α Signalling

In the second part of my thesis I did focus my attention on the role of endogenous NO in the context of the TNF- α signalling pathway.

With the aim of studying the interaction between eNOS and TNF- α signalling, we have generated, in collaboration with Stefania Bulotta (Faculty of Pharmacology; University of Catanzaro, Italy) a line of HeLa cells stably transfected with eNOS cDNA under the control of a doxycycline responsive element that allows a tight regulation of mRNA transcription and protein expression. HeLa cells were chosen for two reasons: because they express TNFR1 and die by apoptosis when treated with TNF- α (**Castellino et al, 1997**) and because they are devoid of any endogenous NOS. In the HeLa system, therefore, the role of eNOS in cell death could be investigated after the introduction of the enzyme in a highly internally regulated model.

As a first step, my research project involved an extensive characterization of the cell line, in order to check both the stability of the system and whether eNOS was correctly and functionally expressed upon doxycycline removal from the culture medium.

By western blot analysis I showed that expression of the protein, absent in the presence of doxycycline, was switched on already 4-6 hours from antibiotic removal, reaching a steady state of expression after 72 hours. The latter was therefore the time point chosen for all successive experiments. The level of expression of eNOS was inversely proportional to the concentration of the antibiotic, with repression occurring with as little of 0.1 ng/ml of doxycycline. The system appears therefore highly controlled in term of expression of the protein. By immunocytochemistry the expressed enzyme was found to be localized mainly at the plasma membrane and in the Golgi area, as indicated in the literature for the endogenous enzyme (**Sessa et al, 1995**).

In terms of enzymatic activity, assayed by the arginine/citrulline assay, HeLa tet-off cells showed a Ca^{2+} -dependent activity in both cellular extract and intact cells, only when cultures were carried out in absence of dox. In these cells the levels are comparable to those in cells endowed with a native endogenous enzyme, such as the endothelial line E2. As expected, dox⁺ cells, not endowed with eNOS expression, don't display this activity. To this eNOS activity corresponded production of bioavailable NO and formation of cGMP, which was introduced in the system by cell reporter experiments with the rat pheochromocytoma cells, PC12.

Taken together, these data indicate that our eNOS, when induced in HeLa cells, retains features of native enzyme, in terms of intracellular localization and enzymatic activity (Sessa et al, 1995; Fulton et al, 1999).

The eNOS Tet-off cells so represent a clean, tightly and internally controlled system to study the eNOS role in TNF signalling and its effect of apoptotic cell death. In fact, any difference between cells cultured with or without doxycycline could be directly accounted to the enzyme expression. In the case of TNF- α induced apoptosis, when the gas was administered together with, or prior to, the cytokine had only been shown either by using either NO donors (see the first part of this thesis and Denadai et al, 2000) or by constitutives fluxes of endogenous NO, generated by expression of inducible NOS introduced by adenoviral gene transfer (Kim et al, 1997; Dimmeler et al, 1997; Li et al, 1999).

The latter studies were inadequate to establish directly whether an effective relationship exist between TNF- α signalling and eNOS, and whether generation of NO represent a physiological built-in system triggered by the apoptotic stimuli itself, in order to modulate its own signalling. To elucidate these aspects I have studied cell death induced by TNF- α in the usual paradigm of cell death, i.e. in the presence of the protein synthesis inhibitor cycloheximide, comparing cells expressing eNOS or not.

Induction of eNOS was able to reduce significantly TNF- α triggered cell death, albeit not abolishing it. Remarkably, the partial protection was removed if cells were treated previously with the NOS inhibitor L-NAME, which in contrast had no effect on the basal level of cell death, indicating that eNOS action depends on the generation of NO upon death stimuli. eNOS expression, therefore, is both necessary and sufficient to protect cells from apoptosis, and this action required generation of NO.

I then investigated whether this effect is due to a tonic, basal level of NO produced by eNOS during the apoptotic stimuli, or is instead due to a 'acute' stimulation of the enzyme by TNF- α . Arginine/citrulline assays and western blot analyses carried out with an antibody able to recognise activated eNOS showed that TNF- α is able to acutely activate the enzyme already in the first minutes of the treatment. These results demonstrate that in cells endowed with eNOS the apoptotic program stimulated by the cytokine is modulated through the autocrine generation of eNOS. Since, however, eNOS activation by TNF- α had not been described yet, thus, I decided to characterize further the signaling mechanism underlying the action of the cytokine.

A 'classical' Ca^{2+} -dependent step does not seem to be involved in this signalling, since TNF- α treatment does not increase $[\text{Ca}^{2+}]_c$. By contrast, eNOS activation correlated with the phosphorylation of Akt on Ser 473 and of eNOS on its Ser 1779, which are post-translational modifications known to activate these enzymes.

Signalling, however, is different from those activated by other stimuli such as: shear stress, estrogen, vascular endothelial growth factor, insulin, sphingosine-1-phosphate, which have been shown to activate eNOS through a Pi3K/AKT-dependent pathway (**Fulton et al, 1999; Dimmeler et al, 1999; Haynes et al, 2000; Fleming et al, 2001; Harris et al, 2001; Igarashi et al, 2001**).

In the case of TNF- α activation of eNOS the difference is clearly indicated by the experiments where I compared the time-course of eNOS activation with those triggered by EGF, a direct activator of Pi3K pathway. This difference concerned the pattern of activation (and inactivation) of eNOS: EGF triggered a rapid activation of eNOS that was already detectable after 2 minutes, both by western blot analysis and by an arginine/citrulline assay. In contrast, activation of eNOS induced by TNF- α occurred with delay of 4-6 minutes, consistent with the delayed activation of Akt .

This delay in eNOS activation suggests that the activation of eNOS by TNF- α is caused not by a direct, but by an indirect event. I therefore decided to analyze SMase role in this context, and this for several reasons: ceramide is generated by TNF- α after few minutes from the stimuli (**Hannun 1996**); data in the literature indicate that in various cell systems ceramide and SMases have a role in the activation of Pi3K/Akt. Remarkably, in all these studies the activation of Akt was shown to initiated 4-5 minutes from the stimuli (**Ibitayo et al, 1998; Hanna et al, 1999; Plo et al, 1999; Huang et al, 2001; Monick et al, 2001**); finally, reports in the literature showed that exogenous short chain analogues of ceramide can activate eNOS (**Igarashi et al, 1999**) while ceramide analogues, added to isolated blood vessels, lead to vasorelaxation, an effect which has classically abscribed to NO (**Johns et al, 1998**).

Data obtained by the using of various SMase inhibitors, support a specific role for N-SMase in the activation of eNOS. In addition the use of N-SMase inhibitors, together with the Pi3K inhibitor wortmannin, allowed us to establish that the eNOS pathway of activation occurs through a sequential involvement first of N-SMase and then of Pi3K/Akt.

This emerged from the following three observations:

- 1) Inhibition of both N-SMase and Pi3K are sufficient to block TNF- α triggered eNOS activation
- 2) Inhibition of N-SMase inhibits Akt activation by TNF- α
- 3) Inhibition of Pi3K does not affect N-SMase activation triggered by TNF- α

We still don't know whether the effects are mediated by a direct action or mediated downstream by a metabolite, such as sphingosine-1-phosphate. The latter had already been already suggested to activate eNOS through a mechanism not fully clarified, which appears to include the activation a type of cell surface receptors, the EDG receptors.

At variance with those obtained with EGF, our results with TNF- α , also show that eNOS activation is switched-off prior to that of Akt. An attractive hypothesis for the time dissociation between Akt and eNOS activation by TNF- α is that the TNF- α /N-SMase mode of activation might include the switching on of a signal transduction pathway capable of selectively dephosphorylating, and thus inactivating, eNOS: this might explain the shorter duration of activation of the enzyme by TNF- α in comparison to a direct stimulus such as EGF, which doesn't involve the activation of SMase. The latter enzyme had indeed been shown to activate protein kinases and phosphatases, including members of the mitogen activated protein kinase family, protein Kinase C- ξ as well as protein phosphatases 1 and 2A (**Kolesnick and Kronke, 1998; Perry and Hannun, 1998; Levade and Jaffrezou, 1999**).

In principle, all of these molecules might be involved in eNOS de-activation, either directly or through an intermediate player. A likely candidate might be protein phosphatase 2A since this enzyme has been proposed to have a specific role in de-phosphorylating eNOS at Ser1179 (**Fleming et al, 2001**). In the future, further studies will address this question, investigating also the consequences in term of cellular signalling, of the different pattern of eNOS activity induced by different stimuli.

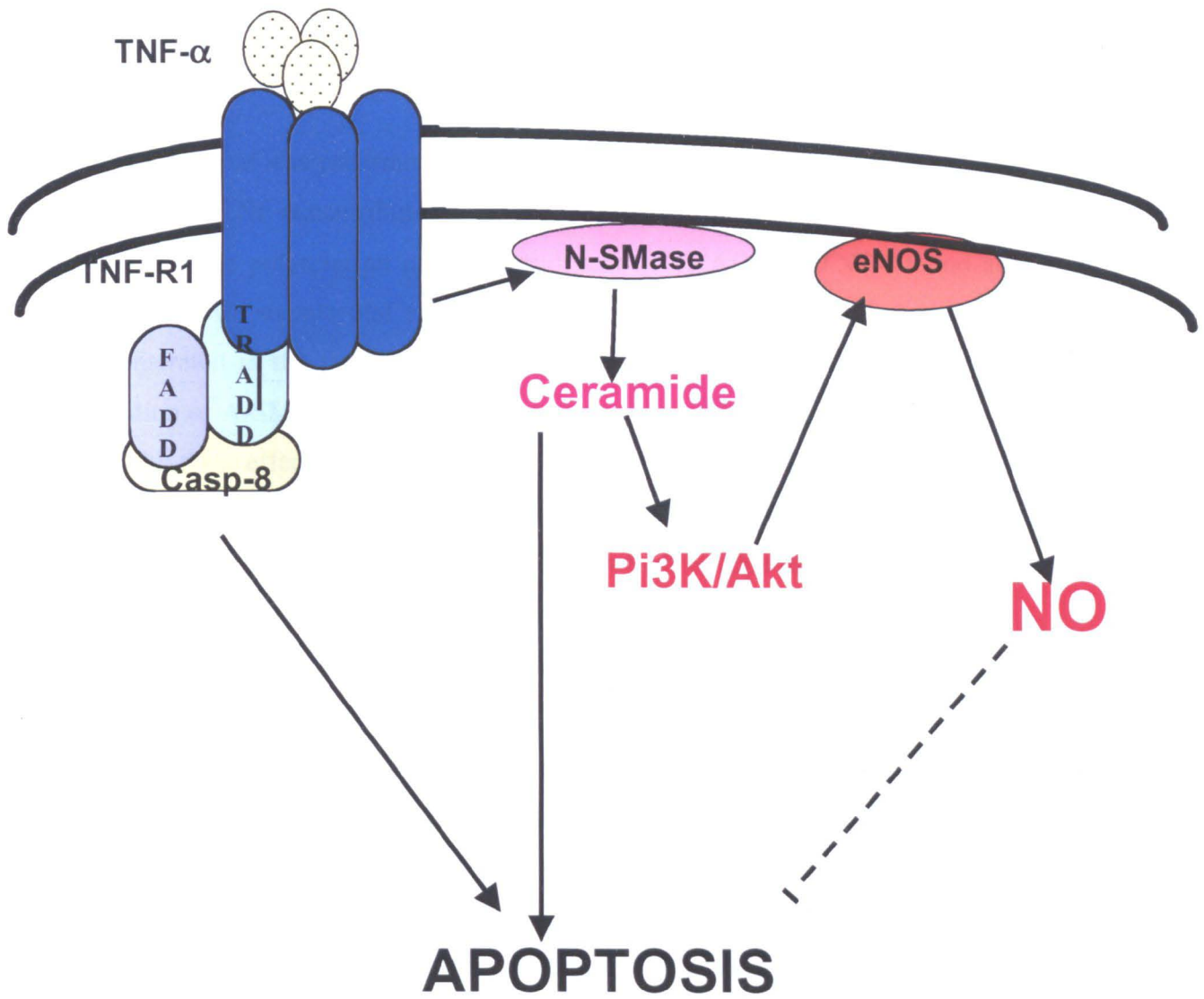


Figure 3.2) TNF- α activates eNOS through a N-SMase/Pi3K/Akt dependent signalling pathway.

3.3) Cross Talk between NO and Ceramide: a Hypothesis

In the work that was preliminary to the first part of this thesis we found that generation of ceramide by TNF- α contributes to apoptosis, at least in part due to a previously unreported effect, i.e. the potentiation of the recruitment of TRADD to TNF-R1 and activation of caspase-8, two events that take place shortly (minutes) after receptor activation. As I demonstrated in the first part of the thesis exogenous NO, through a cGMP-dependent inhibition of A-SMase and of the early wave of ceramide generated by this activity, is able to inhibit these effect of ceramide.

In the second part of the thesis I showed that in cells endowed with a functional eNOS, TNF- α rapidly induces a production of NO, which is dependent on the cytokine-induced activation first of N-SMase and then of Pi3K/Akt.

Tnf-R1, N-SMase and eNOS have all been reported to reside in caveolae at the plasma membrane (Sowa et al, 2001; Veldman et al, 2001). The functional link among them may therefore reflect their morphological association. Experimental evidence suggest that these molecules may interact to constitute a physiological signalling complex active when eNOS is present, as it is in the case of HeLa dox- cells. The link we have shown might have broad significance not only in physiology but also in pathology. A recent report in a rat model of portal hypertensive gastropathy indicates that TNF- α also activates eNOS in endothelial cells (Kawanaka et al, 2002). eNOS and SMases appear therefore to generate a NO/ceramide-based, two-messenger system, triggered by TNF- α to regulate, in a bi-directional way, the initial steps of its own signalling pathway.

The scheme proposed in **Fig 3.3** reports a hypothetical model of TNF- α , SMase and NOS interaction, based on these results. TNF- α , through its class 1 receptor, is able to activate a complex series of signalling partners, among which SMase. Ceramide, generated from A-SMase activity, is able to potentiate proximal events of the apoptotic signalling, such as TRADD recruitment and caspase-8 activation. In parallel, in the presence of a functional eNOS, N-SMase activation induces the generation of lipid messengers that activates the enzyme to produce NO, which therefore will play a protective action on the apoptotic signalling.

In spite of the model, many question remain still open. For example, we do not understand the reason for the dichotomy of effect of the activation of the two SMases, with A-SMase

playing a detrimental and N-SMases playing a protective role in the context of regulation of TNF- α triggered cell death. A possible explanation might be topological, based on the differently localized generation of ceramide (or of its degradation products) with ensuing differential effects.

Additional experiments are thus needed to clarify this point and to reconstitute the cellular signalling system we have unraveled by the use of different cellular and bio-molecular tools, including dominant negative mutants of both form of SMases (as soon as they become available) and of Pi3K. These experiments will permit to assess unambiguously the role of the different SMases isoforms in the context of TNF- α signalling, and to understand better its role in physiology and pathology.

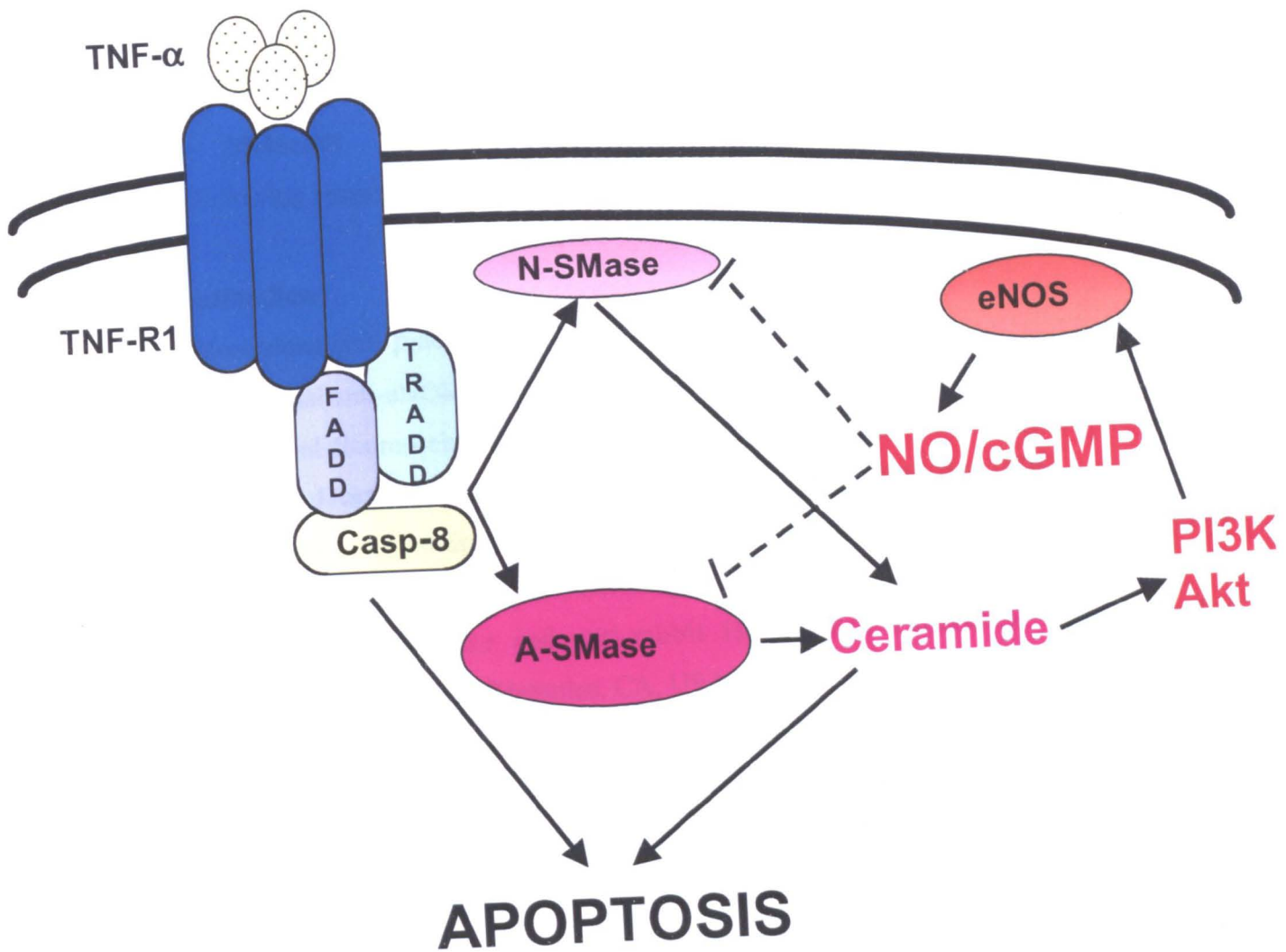


Figure 3.3) Hypothetical model of cross-talk between NO and SMase in regulation of TNF-α triggered apoptosis

Chapter 4

MATERIALS AND METHODS

4.1) Materials

The following reagents were purchased where indicated:

Antibodies:

- Monoclonal anti- β -actin from Sigma (Saint Louis, MS, USA)
- Monoclonal anti-eNOS from Transduction Laboratories (Lexington, United Kingdom)
- Polyclonal fluorescein-labeled anti-mouse IgG and rhodamine-conjugated anti-rabbit IgG from Jackson ImmunoResearch Laboratories (West Grove, PA, USA)
- Polyclonal anti-giantin was a kind gift of Dr. M. Renz (Institute of Immunology and Molecular Genetics, Karlsruhe, Germany)
- Polyclonal anti-mouse and anti-rabbit Horseradish Peroxidase (HRP) coupled antibodies from Biorad (Hercules, CA, USA)
- Polyclonal anti-phospho(Ser 1177)-eNOS, anti-phospho-Akt (Ser 473) and anti-Akt from Cell Signaling Technology (Beverly, MA, USA)
- Polyclonal anti-procaspase-8 and anti-procaspase-3 from Santa Cruz Biotechnology (Santa Cruz, CA, USA)

Reagents:

- [N-methyl- ^{14}C]-sphingomyelin, [^{14}C]-thymidine, L-[^{13}H]-thymidine, [^{32}P]-ATP and the Enhanced Chemiluminescence (ECL) kit from Amersham Corporation (Little Chalfont, United Kingdom)
- Bicinchonic Acid Assay (BCA kit) from Perbio (Bezons, France)
- Bovine eNOS cDNA was a kind gift of Dr. William C. Sessa (Yale University School of Medicine, New Haven, CT, USA)
- C16 ceramide, cycloheximide (CHX), Imipramine, Ly294002, Manumycin A, Monensine, Wortmannin, and all other chemicals were from SIGMA (St.Louis, MO, USA)

- Cell culture media were from GIBCO (Basel, Switzerland) except for foetal clone III, obtained from Hyclone-Celbio (Milan, Italy) and for Tet System-Approved foetal bovine serum (FBS) obtained from Clontech (Palo Alto, CA, USA)
- FITC-labeled Annexin-V from Boehringer Mannheim (Basel, Switzerland)
- Hela Tet-Off cells were from Clontech (Palo Alto, CA, USA)
- N-acetyl-IETD-7-amino-4trifluoromethylcoumarin from Eppendorf (Hamburg, Germany)
- Nitro-cellulose filters from Schleicher & Schuell (Dassel, Germany)
- Oregon green 488 phalloidin from Molecular Probes (Leiden, the Netherlands)
- Protease inhibitor cocktail Complete, Roche Diagnostics (Basel, Switzerland)
- Recombinant human TNF- α , H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) from Alexis (Florence, Italy)
- Scyphostatin was a kind gift of Dr. T Ogita (Sankyo Ltd., Tokyo, Japan)
- Tricyclodecan-9-yl xanthate (D609), S-nitroso-acetylpenicillamine (SNAP), C₂ and C₁₆ ceramide from Calbiochem (Bad Soden, Germany)

4.2) Cell culture and treatments:

U937 cells were routinely grown at 37°C, 5% CO₂, in RPMI medium 1640 supplemented with 10% heat-inactivated foetal clone III serum, 2 mM glutamine, 100 units/ml streptomycin, and 100 units/ml penicillin and used within the 10th week from thawing. All following experiments were carried out in the above culture medium.

HeLa Tet-off cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % Tet System Approved FBS, 50 UI/ml penicillin, 50 µg/ml streptomycin, 2 mM L-glutamine, 200 µg/ml G418, 200 µg/ml Hygromycin and maintained at 37 °C under a 5 % CO₂ atmosphere.

PC12 cells were grown at 37°C, 5% CO₂ in DMEM supplemented with 10% heat-inactivated horse serum and 5% Fetal Clone II.

Preincubations with SNAP (1-300 µM), ODQ (1 µM), 8-Br-cGMP (3 mM), monensin (1 µM), scyphostatin (1 µM), D609 (25 µg/ml), manumycin A (5 µM), L-carnitine (10 µg/ml), wortmannin (100 nM), LY 294002 (5 µM), N^ω-nitro-L-arginine methyl ester (L-NAME;

0.5 mM) were for 15 minutes prior to TNF- α (100 ng/ml), EGF (100 ng/ml) or ATP (100 μ M) administration, or as indicated in the pertinent figure legend; Imipramine pre-treatment (20 μ M) was for 1h. SNAP was used at concentrations inducing no cytotoxicity effects and D609 under conditions not stimulating ceramide generation (DeNadai et al, 2000)

Cells were incubated in the presence of the various compounds throughout the experiments.

4.3) Measurement of SMase activities in U937 cells

SMase assays were performed essentially as reported in Wiegmann et al, 1994. Following pre-incubation with various drugs, cell samples (2×10^6 cells) were treated with TNF- α (50 ng/ml at 37°C). Incubations were stopped at the indicated time-points by immersion of reaction tubes in a methanol/dry ice bath. Cells were then centrifuged (1000 g for 5 min at 4°C) and the pellet washed once with ice-cold phosphate buffered saline.

To assay the A-SMase activity cell pellets were resuspended in 0.2 ml of 0.1 % Triton X-100 at 4°C for 15 min and subsequently homogenised by repeated squeezing through an 18-gauge needle. Homogenates were then centrifuged (1000 g for 5 min at 4°C) in order to remove nuclei and cellular debris, and the supernatant was incubated for 2 h at 37°C in a buffer containing: 250 mM Na acetate, 1 mM EDTA, [N-methyl- 14 C]-sphingomyelin (55 mCi/mmol; 50000 dpm/assay), pH 5.5.

To assay N-SMase activity, cell pellets were resuspended in 0.2 ml of a buffer consisting of: 0.2 % Triton X-100, 20 mM HEPES, 10 mM MgCl₂, 2 mM EDTA, 5 mM dithiothreitol, 0.1 mM Na₃VO₄, 30 mM p-nitrophenylphosphate, 10 mM β -glycerophosphate, 750 μ M ATP, 1 mM phenylmethylsulfonyl fluoride, 10 mM leupeptin, 10 mM pepstatin, 5 mg/ml soybean trypsin inhibitor and 2 mg/ml aprotinin, pH 7.4. After 5 minutes incubation on ice, cells were homogenised as above. Cell lysates were then incubated for 2 h at 37°C in a buffer containing 20 mM HEPES, 1 mM MgCl₂ and [N-methyl- 14 C]-sphingomyelin (55 mCi/mmol; 50000 dpm/assay), pH 7.4.

Reactions for both A-SMase and N-SMase were stopped by addition of 250 μ L of ice-cold CHCl₃:MeOH (2:1, vol:vol) and 250 μ L of ice-cold H₂O. Phosphorylcholine thus produced was separated from sphingomyelin by sequential washing with 800 μ L of ice-cold

CHCl₃:MeOH (2:1, vol:vol), 500 µL of CHCl₃ and 750 µL of CHCl₃:MeOH:H₂O (2:47:48, vol:vol:vol).

Aqueous and organic phases were then collected separately and radioactive phosphorylcholine in the aqueous phase counted by liquid scintillation in a Beckman β-counter. Non-hydrolysed, radioactive sphingomyelin in the organic phase was routinely measured as an internal control. To check for the specificity of the assay conditions samples containing either purified A-SMase or N-SMase (Sigma) were assayed in parallel as positive controls. Results were normalised to the protein content evaluated by the BCA assay.

4.4) Apoptosis detection in U937 cells

4.4a) Western blot analyses of procaspase-8 and 3 in U937:

Cell lysates were prepared from aliquots of 2×10^6 cells. After pertinent treatments, cell aliquots were put on ice, washed rapidly twice with 150 mM NaCl, 1 mM EDTA, 2 mM Na₂P₂O₆, 30 mM NaF, 20 mM Hepes, pH 7.5 and lysed in the same buffer containing 1% Triton X-100, 0.1 mM phenylmethylsulphonylfluoride, 10 mg/ml leupeptin and 10 mg/ml aprotinin. Lysates were centrifuged at 3000 rpm for 5 minutes and the supernatants were transferred to other vials. Protein concentration of the samples was assessed by using the BCA procedure.

The Western blot analyses were carried out on 30 mg of soluble proteins separated on 12% SDS-PAGE and transferred onto nitro-cellulose membranes. Nonspecific binding was blocked before immunolabeling by incubation for 1 h at room temperature with blotting buffer (PBS containing 0.1% Tween and 5% nonfat milk). The membranes were incubated overnight in blotting buffer supplemented with the anti-procaspase-8; after this incubation membranes were washed for 1 h with blotting buffer. The secondary HRP labelled antibody then was added for 30 min. After several washes of the membranes in blotting buffer, the antigens were revealed by autoradiography with ECL according to the manufacturer's instructions. As an internal control, the membranes were subsequently stripped off of the first antibody and reprobbed with the antibody recognising procaspase-3. Stripping was carried out by incubating nitrocellulose filters for 30 min at 52°C with a buffer consisting

of: 62.5 mM Tris-HCl, 2% SDS, 1% β -mercaptoethanol, pH 6.7; filters were then washed extensively in 100 mM Tris-HCl, 0.1% Tween 20, pH 7.4, before equilibration for 1h in blotting buffer and reprobing of the membrane with the appropriate antibody.

4.4b) Measurement of caspase-8 activity in U937 cells

Aliquots of 2×10^6 U937 cells, pre-incubated with the various drug treatments described above, were treated for 5 minutes with TNF- α (50 ng/ml) in the presence of CHX (1 μ g/ml), washed in ice cold 150 mM NaCl, 1 mM EDTA, 2 mM Na₂P₂O₆, 30 mM NaF, 20 mM HEPES, pH 7.5 and lysed in the same buffer containing 1 % Triton X 100, 0.1 mM phenylmethylsulphonylfluoride, 10 μ g/ml leupeptin and 10 μ g/ml aprotinin. Protein concentration of the samples was assessed by BCA assay.

100 μ g of soluble proteins were then diluted in a reaction buffer containing a final concentration of 25 mM HEPES, 100 mM NaCl, 5 mM dithiothreitol, 1 mM EDTA, 0.1% CHAPS and 10% sucrose. Caspase-8 activity was assayed in a Perkin-Elmer LS50 fluorimeter, equipped with 400 nm excitation and 505 nm emission filters, by measuring for 1 h at 37 °C the fluorescence emitted by the cleaved specific caspase-8 substrate, N-acetyl-IETD-7-amino-4-trifluoromethylcoumarin (25 μ M), calibrated with respect to a 7-amino-4-trifluoromethylcoumarin concentration curve

4.4c) DNA fragmentation

Secondary DNA fragmentation following TNF- α /CHX treatment was quantitatively measured as described in Sestili et al, 1996. Cells were labelled overnight with [¹⁴C]-thymidine (0.05 μ Ci/ml) and incubated for a further 6 h in a medium containing unlabelled thymidine (1 μ g/ml) before pre-incubation with or without the various compounds and treatment with TNF- α (50 ng/ml) plus CHX (1 μ g/ml). At the indicated time-points the cells were sedimented (1000 rpm, 5 min at 4°C) and the pellet was resuspended in a buffer solution containing: 140 mM NaCl, 5 mM KCl, 5 mM glucose, 5 mM EDTA, 4 mM NaHCO₃, pH 8.3. Aliquots of 0.5×10^6 cells were then loaded onto protein adsorbing (polyvinylchloride) filters (25 mm, 2 μ m pore), washed with the same buffer and lysed with a solution containing 0.2 % sarkosyl, 2 M NaCl and 40 mM EDTA, pH 10.1. Filters were

rinsed with EDTA (20 mM, pH 10.1) and then removed from the filter holders which were washed twice with 0.4 N NaOH. Radioactivity was counted in the lysates, EDTA washes, filters and filter holder washes. For each sample, the DNA fragmentation was determined as the percentage of the ^{14}C -labeled DNA recovered in the lysate plus the EDTA washes.

4.4d) Annexin-V staining

Cells, pretreated and exposed to TNF- α as indicated in the previous sections were analyzed for phosphatidylserine exposure at the external surface of the plasma membrane.

Aliquots of 5×10^4 cells were stained with fluorescein isothiocyanate-labeled (FITC)-Annexin V (0.5 mg/ml in phosphate buffered saline) for 15 minutes at room temperature, after the indicated TNF- α time treatment and with the various indicated pretreatment. Single cell Annexin V positive staining was then evaluated using a fluorescence-activated cell sorter (FACStar Plus, Becton Dickinson, Sunnyvale, CA, USA)

4.5) Transfection and selection of eNOS conditionally expressing HeLa cells

Cells grown on 35 mm Petri dishes to $\sim 40\%$ confluence were co-transfected by the calcium phosphate method (DeSilvestris et al, 1995) using 0.5 μg of pTRE/eNOS plasmid and 25 ng of pTK-Hygr plasmid (Clontech) per cm^2 of culture dish. Three days after transfection, the cells were detached with trypsin and plated on Petri dishes in the presence of doxycycline hydrochloride (0.01-1 $\mu\text{g}/\text{ml}$) and hygromycin B (200 $\mu\text{g}/\text{ml}$). After 3 weeks, hygromycin-resistant colonies were expanded in 24-well Petri dishes. Stable transfectants were maintained in medium supplemented with 100 $\mu\text{g}/\text{ml}$ G418, 100 $\mu\text{g}/\text{ml}$ hygromycin and 1 $\mu\text{g}/\text{ml}$ doxycycline.

To induce expression of eNOS, cells were plated in the absence of doxycycline: unless otherwise specified, experiments were carried out on cells cultured for 72 h with or without the antibiotic.

4.6) Western blot analysis in HeLa cells

After the various treatments, cells monolayers in the presence or absence of doxycycline were washed free of medium and solubilised by direct addition of a pre-heated (to 80°C) denaturing buffer, containing 50 mM Tris-Cl pH 6.8, 2% SDS and a protease inhibitor cocktail. Solubilised samples were collected and immediately boiled for 2 min. Bromophenol blue, glycerol and β -mercaptoethanol were then added to final concentrations of 0.05%, 10% and 2%, respectively.

Samples were boiled again before loading onto 10% SDS-polyacrylamide gels. After electrophoresis, polypeptides were electrophoretically transferred to nitro-cellulose filters. Monoclonal anti-eNOS (dilution 1:1000 in 3%BSA/PBS), polyclonal anti-phospho eNOS (dilution 1:1000 in 3%BSA/PBS), monoclonal anti-Akt (dilution 1:1000 in 3%BSA/PBS), polyclonal anti-phospho Akt (dilution 1:1000 in 3%BSA/PBS) and monoclonal anti- β -actin (dilution 1:1000 in 3%BSA/PBS) antibodies were used to reveal the respective antigens. After incubation with appropriate secondary reagent, the antigens were revealed by autoradiography with ECL procedure.

4.7) Immunofluorescence in HeLa cells

HeLa cells plated on glass coverslips and grown in the presence or absence of doxycycline, were fixed with 4% paraformaldehyde in 120 mM $\text{Na}_2\text{P}_2\text{O}_6$, pH 7.4, for 30 min at 37°. The monolayers were then permeabilized with Triton X-100 and processed as described in **De Silvestris et al (1995)**. In some experiments cells were immunostained with only monoclonal anti-eNOS antibody; in others they were doubly immunostained with monoclonal anti-eNOS antibody (dilution 1:100) and polyclonal anti-giantin antibody (dilution 1:100), to stain the Golgi complex. Bound monoclonal and polyclonal antibodies were then revealed by fluorescein-labelled anti-mouse IgG and rhodamine-conjugated anti-rabbit IgG.

To reveal actin filaments, cells were stained with a fluorescent derivative of phalloidin toxoid, Oregon green 488 phalloidin (Molecular Probes): in this case the eNOS immunostaining was revealed by the use of a rhodamine-conjugated anti-mouse IgG.

Coverslips were observed under a NIKON Optiphot 2 microscope equipped for epifluorescence or with a BIO-RAD MRC 1024 laser confocal microscope.

4.8) Measurement of $[Ca^{2+}]_c$ in HeLa cells

Cells were detached by trypsinisation, washed in a Krebs Ringer HEPES buffer containing 125 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4 , 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 6 mM glucose, 25 mM HEPES-NaOH, pH 7.4, and loaded in the same buffer with the Ca^{2+} -sensitive dye, fura-2 (4.5 μ M), administered as acetoxymethylester for 30 min at 25°C.

Cell preparations were then diluted (5×10^6 cells) and $[Ca^{2+}]_c$, before and after challenge with either ATP, EGF or TNF- α , was analysed in a Perkin Elmer LS-50B fluorimeter as described (Clementi et al, 1995).

4.9) NOS activity assays in HeLa cells

NOS activity was quantified by measuring the conversion of L- $[^3H]$ arginine into L- $[^3H]$ citrulline in both homogenised and intact cells. In the first approach, cells cultured in the presence or absence of doxycycline were detached by trypsinisation, pelleted and resuspended (2×10^7 cells/ml) in an homogenisation buffer containing: 320 mM sucrose, 1 mM dithiothreitol, 1 mM EDTA, 20 mM HEPES, pH 7.2, supplemented with a protease inhibitor cocktail. After homogenisation by sonication (30 sec at 4°C), 50 μ l of cell extract were added to 100 μ l of a reaction buffer containing: 1.2 mM $MgCl_2$, 10 μ M tetrahydrobiopterin, 1 μ M FAD, 1 μ M FMN, 1 μ M NADPH, 0.45 mM $CaCl_2$, 10 μ g/ml calmodulin, 50 μ M L- $[^3H]$ arginine (0.8 μ Ci/sample) and 25 mM Tris, pH 7.4. The reaction was carried out for 10 minutes at 37°C and stopped by the addition of 2 ml of 50 mM HEPES, pH 5.5, supplemented with 5 mM EGTA. Samples containing either N $^{\omega}$ -monomethyl-L-arginine (L-NMMA; 500 μ M) or EGTA (1 mM) in the reaction buffer were processed in parallel as controls.

NOS activity in intact cells following pre-incubation with various compound as indicated in the section on *Cell culture and treatments*, was instead measured on monolayers washed and then incubated for 20 min at 37°C with a reaction buffer containing, in addition to the

inhibitors: 145 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 10 mM glucose, 1 mM CaCl₂ and 10 mM HEPES, pH 7.4.

In the experiments measuring eNOS activation by TNF- α , ATP or EGF, 2.5 μ Ci/ml of L-[³H] arginine was added 1 min before stimulation with the cytokine.

In the experiments aimed at comparing the kinetics of eNOS activation by TNF- α , ATP or EGF we used the same protocol except for the fact that 2.5 μ Ci/ml of L-[³H] arginine was added at various time-points after administration of each eNOS activating compound and the reaction stopped after 2 min. Non-stimulated cells were always run in parallel as controls.

The reaction was then stopped at the various indicated time points by washing the monolayers with 2 ml of ice-cold phosphate buffered saline, pH 7.4, supplemented with L-arginine (5 mM) and EDTA (4 mM). 0.5 ml of 100% cold ethanol was added to the dishes and left to evaporate before a final addition of 2 ml of 20 mM HEPES, pH 6.0, to resuspend the formed radioactive product (L-Citrulline) as well as the unconverted radioactive substrate. The separation of L-[³H] citrulline from L-[³H] arginine was obtained by DOWEX 50X8-400 chromatography as described in **Bredt and Snyder, (1989)**.

In the assay carried out with homogenised cells, values obtained from samples incubated without cell extracts were subtracted. Data in the intact cells are presented without background correction. L-[³H] citrulline formed was normalised to protein content as determined by the BCA assay.

4.10) cGMP measurements in HeLa cells

NO-dependent guanosine 3',5'-cyclic monophosphate (cGMP) generation by the transfected HeLa clone Ad was measured in both the cells themselves and in a concubation system using PC12 cells as a reporter system: samples of suspended 2x10⁶ HeLa cells and 0.5x10⁶ cells, either separate or together, were treated for 15 minutes at 37°C in the presence or absence of either ATP (100 μ M), TNF- α (100 ng/ml) or S-nitrosoacetylpenicillamine (100 μ M) in 1 ml of a solution containing 125 mM NaCl, 5 mM KCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 2mM CaCl₂, 6 mM glucose, 1 mM L-arginine, 0.6 mM isobuthylmethylxanthine (IBMX) and 25 mM HEPES, pH 7.4, with or without L-

NAME (0.5 mM). The reaction was terminated by addition of ice-cold trichloroacetic acid (final concentration 6%). After ether extraction, cGMP levels were measured using a radioimmunoassay kit (Perkin Elmer Life Sciences) and normalized to cellular proteins with the BCA assay.

4.11) Apoptosis detection in HeLa cells

Apoptosis was measured by flow cytometry using two different protocols as described in Sciorati et al, 1997. In the first approach, phosphatidyl serine exposure was monitored by staining for 15 min at room temperature with FITC-labelled annexin V (0.5 µg/ml in phosphate buffered saline). The loss of the ability to exclude vital dyes was assessed using propidium iodide in an isotonic buffer (10 mg/ml in phosphate buffered saline). Early apoptotic cells exclude the dye but are stained with annexin V, whereas non apoptotic cells are negative for both.

In the second approach the hypodiploid DNA peak in single parameter DNA histograms typical of apoptotic cells was identified. To this end DNA was stained in unfixed cells incubated for 60 min at 37°C in sodium citrate 0.1%, propidium iodide (50 mg/ml), RNase A (100 µg/ml; Sigma) and Nonidet P40 (0.01%).

Cells were analysed for either DNA content or annexin V staining using a fluorescence-activated cell sorter (FACStar Plus, Becton Dickinson, Sunnyvale, CA).

4.12) Statistical analysis

The results are expressed as means ± standard error of the mean (s.e.m.); *n* represents the number of individual experiments. Statistical analysis was carried out using the Student's *t* test for unpaired variables (two-tailed). The asterisks *,** or *** or by +, ++ and +++ refer to statistical probabilities (*P*) of < 0.5, < 0.01 and < 0.001, as indicated in the various legend to figures or tables.

REFERENCES

- Abu-Soud HM and Stuhler DJ** (1993): Nitric Oxide synthases reveal a role for calmodulin in controlling electron transfer. *Proc. Natl. Acad. Sci. USA.* 90: 10769-10772
- Adam D, Wiegmann K, Adam-Klages S, Ruff A and Kronke M** (1996): A novel cytoplasmic domain of the p55 tumor necrosis factor receptor initiates the neutral sphingomyelinase pathway. *J Biol Chem.* 271: 14617-14622
- Adam-Klages S, Adam D, Wiegmann K, Struve S, Kolanus W, Schneider-Mergener J and Kronke M** (1996): FAN, a novel WD-repeat protein, couples the p55 TNF-receptor to neutral sphingomyelinase. *Cell.* 86: 937-947
- Adams JM and Cory S** (1998): The Bcl-2 protein family: arbiters of cell survival. *Science* 281: 1322-1326
- Albina JE, Cui S, Mateo RB and Reichner JS** (1993): Nitric oxide mediates apoptosis in murine peritoneal macrophages. *J. Immunol.* 150: 5080-5085
- Alessi DR, Andjelkovic M, Caudwell C, Cron P, Morrice N, Cohen P and Hemmings BA** (1996): Mechanism of activation of protein kinase B by insulin and IGF-1. *EMBO J.* 15: 6541-8551
- Alnemri ES, Livingston DJ, Nicholson DW, Salvesen G, Thornberry NA, Wong WW and Yuan J.** (1996): Human ICE/CED-3 protease nomenclature. *Cell* 87, 171
- An S, Goetzl EJ and Lee H** (1998): Signaling mechanisms and molecular characteristics of G protein-coupled receptors for lysophosphatidic acid and sphingosine 1-phosphate. *J Cell Biochem Suppl.* 30-31: 147-157
- Andrieu-abadie N, Gouazè V, Salvayre R and Levade T** (2001): Ceramide in apoptosis signaling: relationship with oxidative stress. *Free Radic. Biol. Med.* 31: 717-728
- Andrieu-Abadie N, Jaffrézou JP, Hatem S, Laurent G, Levade T and Mercaider JJ** (1999): L-carnitine prevents doxorubicin-induced apoptosis of cardiac myocytes: role of inhibition of ceramide generation. *FASEB J.* 13:1501-1510
- Aravind L, Dixit VM and Koonin** (1999): The domains of death: evolution of the apoptosis machinery. *Trends Bio. Sci.* 24: 47-53
- Arenz C, Thutewohl M, Block O, Waldmann H, Altenbach HJ and Giannis A** (2001): Manumycin A and its analogues are irreversible inhibitors of neutral sphingomyelinase. *Chembiochem.* 2:141-143
- Armstrong RC, Aja TJ, Hoang KD, Gaur S, Bai X, Alnemri ES, Litwack G, Karanewsky DS, Fritz LC and Tomaselli KJ** (1997): Activation of the CED3/ICE-related protease CPP32 in cerebellar granule neurons undergoing apoptosis but not necrosis. *J. Neurosci.* 17: 553-562

- Arnold WP, Mittal CK, Katsuki S, and Murad (1977):** Nitric oxide activates guanylate cyclase and increases guanosine 3'-5'-cyclic monophosphate levels in various tissue preparations. *Proc. Natl. Acad. Sci. USA.* 74: 3203-3207
- Basu S, Bayoumy S, Zhang Y, Lozano J and Kolesnick R (1998):** BAD enables ceramide to signal apoptosis via Ras and Raf-1. *J Biol Chem.* 273: 30419-30426
- Beg AA and Baltimore D (1996):** An essential role for NF-kB in preventing TNF-alpha-induced cell death. *Science* 274:782-784
- Beltran B, Mathur A, Duchen MR, Erusalimsky JD and Moncada S (2000):** The effect of nitric oxide on cell respiration: A key to understanding its role in cell survival or death. *Proc Natl Acad Sci U S A.* 97: 14602-14607
- Berridge MJ (1984):** Inositol trisphosphate and diacylglycerol as second messengers. *Biochem J.* 220: 345-360
- Bezombes C, Maestre N, Laurent G, Levade T, Bettaieb A, Jaffrezou JP (1998):** Restoration of TNF-alpha-induced ceramide generation and apoptosis in resistant human leukemia KG1a cells by the P-glycoprotein blocker PSC833. *FASEB J.* 12: 101-109
- Bolanos JP, Peuchen S, Heales SJ, Land JM and Clark JB (1994):** Nitric oxide-mediated inhibition of the mitochondrial respiratory chain in cultured astrocytes. *J Neurochem* 63: 910-916
- Boldin MP, Goncharov TM, Goltsev YV and Wallach D (1996):** Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. *Cell.* 85:803-815
- Bonfoco E, Zhivotosky B, Rossi AD, Santelines MA, Orrhenius S, Lipton SA and Nicotera P (1997):** Bcl-2 delays apoptosis and PARP cleavage induced by NO donors in GT1-7 cells. *Neuroreport* 8: 272-276
- Borderie D, Hilliquin P, Hernvan A, Lemarechal H, Menkes CJ and Ekindjann OG (1999):** Apoptosis induced by nitric oxide is associated with nuclear p53 protein expression in cultured osteoarthritic synoviocytes. *Osteoarth. Cartilage* 7:203-213
- Boyd JM, Malstrom S, Subramanian T, Venkatesh LK, Schaeper U, Elangovan B, D'Sa-Eipper C and Chinnadurai G (1994):** Adenovirus E1B 19 kDa and Bcl-2 proteins interact with a common set of cellular proteins. *Cell.* 79:341-351.
- Bredt D and Snyder S (1989):** Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. *Proc. Natl. Acad. Sci. USA.* 86: 9030-9033
- Bredt DS and Snyder SH (1990):** Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proc. Natl. Acad. Sci. USA* 87: 682-685
- Bredt DS, Hwang PM, Glatt CE, Lowenstein C, Reed RR and Snyder SH (1991):** Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. *Nature* 351: 714-718

- Brenman JE, Chao DS, Gee SH, McGee AW, Craven SE, Santillano DR, Wu Z, Huang F, Xia H, Peters MF, Froehner SC and Bredt DS** (1996): Interaction of nitric oxide synthase with the post-synaptic density protein PSD-95 and α -1-syntrophin mediated by PDZ domains. *Cell* 84: 757-767
- Brenman JE, Chao DS, Xia H, Aldape K and Bredt DS** (1995): Nitric oxide synthase complexed with dystrophin and absent from skeletal muscle sarcolemma in Duchenne muscular dystrophy. *Cell* 82: 743-752
- Brenner B, Koppenhoefer U, Weinstock C, Linderkamp O, Lang F and Gulbins E** (1997): Fas- or Ceramide-induced Apoptosis Is Mediated by a Rac1-regulated Activation of Jun N-terminal Kinase/p38 Kinases and GADD153. *J. Biol. Chem.* 272: 22173-22181
- Brockhaus F and Brune B** (1999): P53 accumulation in apoptotic macrophages is an energy dependent dependent process that precedes cytochrome c release in response to nitric oxide. *Oncogene* 18:6403-6410
- Brown GC** (1999): Nitric oxide and mitochondrial respiration. *Biochim. Biophys. Acta* 1411: 351-369
- Cai Z, Bettaieb A, Mahdani NE, Legres LG, Stancou R, Masliah J and Chouaib S.** (1997): Alteration of the sphingomyelin/ceramide pathway is associated with resistance of human breast carcinoma MCF7 cells to tumor necrosis factor-alpha-mediated cytotoxicity. *J Biol Chem.* 272:6918-6926
- Callsen D and Brune B** (1999): Role of mitogen-activated protein kinases in S-nitrosoglutathione-induced macrophage apoptosis. *Biochemistry* 39: 2279-2286
- Castedo M, Hirsch T, Susin SA, Zamzami N, Marchetti P, Macho A and Kroemer G** (1996): Sequential acquisition of mitochondrial and plasma membrane alterations during early lymphocyte apoptosis. *J Immunol.* 157: 512-521
- Castellino AM, Parker GJ, Boronenkov IV, Anderson RA and Chao MV** (1997): A novel interaction between the juxtamembrane region of the p55 tumor necrosis factor receptor and phosphatidylinositol-4-phosphate 5-kinase. *J Biol Chem* 272: 5861-5870
- Cecconi F, Alvarez-Bolado G, Meyer BI, Roth KA and Gruss P** (1998): Apaf1 (CED-4 homologue) regulates programmed cell death in mammalian development. *Cell* 94: 727-737
- Chang HY and Yang X** (2000): Proteases for cell suicide: functions and regulation of caspases. *Microbiol. Mol. Bio. Rev.* 64: 821-846
- Chao W and Olson MS** (1993): Platelet-activating factor: receptors and signal transduction. *Biochem J.* 292:617-629
- Chaudary D, O'Rourke K, Chinnayan AM and Dixit V** (1998): The death inhibitory molecules CED-9 and CED-4L uses a common mechanism to inhibit the CED-3 death protease. *J. Biol. Chem.* 273: 17708-17712

- Chen F, Bradley MH, Conradt B, Zhou Z, Riemer D, Gruenbaum Y and Horvitz HR** (2000). Translocation of *C. elegans* CED-4 to nuclear membranes during programmed cell death. *Science* 287: 1485-1489
- Chinnaiyan AM, O'Rourke K, Lane BR and Dixit VM** (1997): Interaction of CED-4 with CED-3 and CED-9: a molecular framework for cell death. *Science* 275: 1122-1126
- Chinnaiyan AM, O'Rourke K, Tewari M and Dixit VM** (1995): FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. *Cell* 81:505-512
- Cifone M, Roncaioli P, DeMaria R, Camarda G, Santoni A, Ruberti G and Testi R** (1995): Multiple pathways originate at the Fas/APO-1 (CD95) receptor: sequential involvement of phosphatidylcholine-specific phospholipase C and acidic sphingomyelinase in the propagation of the apoptotic signal. *EMBO J.* 14: 5859-5868
- Clementi E, Sciorati C and Nisticò G** (1995) Growth factor-induced Ca²⁺ responses are differentially modulated by nitric oxide via activation of a cyclic GMP-dependent pathway. *Mol. Pharmacol.* 48:1068-1077
- Clementi E, Brown GC, Feelisch M and Moncada S** (1998): Persistent inhibition of cell respiration by nitric oxide: crucial role of S-nitrosylation of mitochondrial complex I and protective action of glutathione. *Proc Natl Acad Sci USA.* 95: 7631-7636
- Cohen GM** (1997): Caspases: the executioner of apoptosis. *Biochem. J* 326: 1-16
- Cory S and Adams JM** (2002): The Bcl-2 family: regulators of the cellular life-or-death switch. *Nat Rev Canc* 2: 647-656
- Corson MA, James NL, Latta SE, Nerem RM, Berk BC and Harrison DG** (1996): Phosphorylation of endothelial nitric oxide synthase in response to fluid shear stress. *Circ Res.* 79: 984-991
- Crompton M** (1999): The mitochondrial permeability transition pore and its role in cell death. *Biochem. J.* 341: 233-249
- Cuvillier O, Pirianov G, Kleuser B, Vanek PG, Coso OA, Gutkind S and Spiegel S** (1996): Suppression of ceramide-mediated programmed cell death by sphingosine-1-phosphate. *Nature* 381: 800-803
- Daniel LW, Sciorra VA and Ghosh S** (1999): Phospholipase D, tumor promoters, proliferation and prostaglandins. *Biochim Biophys Acta.* 1439: 265-276
- Dawson TM, Dawson VL, and Snyder SH** (1992): A novel neuronal messenger in brain: The free radical, nitric oxide. *Ann.Neurol.* 32: 297-311
- De Silvestris M, D'Arrigo A and Borgese N** (1995): The targeting information of the mitochondrial outer membrane isoform of cytochrome b5 is contained within the carboxyl-terminal region. *FEBS Lett.* 370: 69-74

- Decaudin D, Geley S, Hirsch T, Castedo M, Marchetti P, Macho A, Kofler R and Kroemer G** (1997): Bcl-2 and Bcl-XL antagonize the mitochondrial dysfunction preceding nuclear apoptosis induced by chemotherapeutic agents. *Cancer Res.* 57: 62-67
- DeNadai C, Sestili P, Cantoni O, Lievremont JP, Sciorati C, Barsacchi R, Moncada S, Meldolesi J and Clementi E** (2000): Nitric oxide inhibits tumor necrosis factor α induced apoptosis by reducing the generation of ceramide. *Proc. Natl. Acad. Sci. USA* 97: 5480-5485
- Denninger JW and Marletta MA** (1999): Guanylate cyclase and the .NO/cGMP-signaling pathway. *Biochim. Biophys. Acta* 1411: 334-350
- Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R and Zeiher AM** (1999): Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature*. 399: 601-605
- Dimmeler S, Haendeler J, Nehls M and Zeiher AM** (1997): Suppression of apoptosis by nitric oxide via inhibition of interleukin-1 β -converting enzyme (ICE)-like and cysteine protease protein (CPP)-32-like proteases. *J. Exp. Med.* 185: 601-607
- Dimmeler S, Haendeler J, Sause A and Zeiher AM** (1998): Nitric oxide inhibits APO-1/Fas-mediated cell death. *Cell Growth Differ.* 9: 415-422
- Dimmeler S, Hermann C, Galle J and Zeiher** (1999): Up-regulation of superoxide dismutase and nitric oxide synthase mediates the apoptosis suppressive effects of shear stress on endothelial cells. *Arterioscl. Thromb. Vasc. Biol.* 19: 656-664
- Dijkmans R and Billiau A** (1991): Interferon-gamma/lipopolysaccharide-treated mouse embryonic fibroblasts are killed by a glycolysis/L-arginine-dependent process accompanied by depression of mitochondrial respiration. *Eur J Biochem.* 202:151-159
- Duan H and Dixit VM** (1997): RAIDD, a novel death adaptor molecule. *Nature* 377: 446-448
- Earnshaw WC, Martins LM and Kaufmann SH** (1999): Mammalian caspases: structure, activation, substrates and functions during apoptosis. *Annu. Rev. Biochem* 68: 383-424
- Ekert PG, Silke J and Vaux DL** (1999): Caspase inhibitors. *Cell Death Differ.* 6: 1081-1086
- Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A and Nagata S** (1998): A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature* 391: 43-50
- Estevez AG, Spear N, Thompson JA, Cornwell TL, Radi R, Barbeito L and Beckman JS** (1998): Nitric oxide dependent production of cGMP supports the survival of rat embryonic motor neurons cultured with brain derived neurotrophic factors. *J. Neurosci.* 18: 3708-3714

- Evan G and Littlewood TD** (1998): A Matter of Life and Cell Death *Science* 281,1317-1322
- Fanger GR, Gerwins P, Widmann C, Jarpe MB and Johnson GL** (1997): MEKKs, GCKs, MLKs, PAKs, TAKs, and tpls: upstream regulators of the c-Jun amino-terminal kinases? *Curr Opin Genet Dev.* 1: 67-74
- Farinelli SE, Park DS and Greene LA** (1996): Nitric oxide delays the death of trophic factor deprived PC12 cells and symphatetic neurons by a cGMP-mediated mechanism. *J. Neurosci.* 16: 2325-2334
- Farschon DM, Couture C, Mustelin T and Newmeyer DD** (1997): Temporal phases in apoptosis defined by the actions of Src homology 2 domains, ceramide, Bcl-2, interleukin-1beta converting enzyme family proteases, and a dense membrane fraction. *J Cell Biol.* 137: 1117-1125
- Feldman PL, Griffith OW and Stuehr DJ** (1993): The surprising life of nitric oxide. *Chem. Eng. News* 20: 26-30
- Feron O, Dessy C, Moniotte S, Desager JP and Balligand JL** (1999): Hypercholesterolemia decreases nitric oxide production by promoting the interaction of caveolin and endothelial nitric oxide synthase. *J. Clin. Invest.* 103: 897-905
- Firestein BL & Bredt DS** (1999): Interaction of neuronal nitric oxide synthase and phosphofructokinase-M. *J.Biol. Chem* 274: 10545-10550
- Fleming I, Fisshalter B, Dimmeler S, Kemp BE and Busse R** (2001): Phosphorylation of Thr (495) regulates Ca²⁺/Calmodulin dependent endothelial nitric oxide synthase activity. *Circ. Res.* 88: E68-E75
- Fulton D, Gratton JP and Sessa WC** (2001): Post-translational control of endothelial nitric oxide synthase: why isn't calcium/calmodulin enough? *J Pharmacol Exp Ther.*299: 818-824
- Fulton D, Gratton JP, McCabe TJ, Fontana J, Fujio Y, Walsh K, Franke TF, Papapetroupolos A and Sessa WC** (1999): Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* 399: 597-601
- Furchgott RF and Zawadzki JV** (1980): The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 288: 373-376
- Furchgott RF** (1988): Studies on relaxation of rabbit aorta by sodium nitrite: the basis for the proposal that the acid-activatable inhibitory factor from bovine retractor penis is inorganic nitrite and the endothelium-derived relaxing factor is nitric oxide. In *Vasodilation: Vascular Smooth Muscle, Peptides, Autonomic Nerves, and Endothelium*, ed. PM Vanhoutte, pp.401-14. New York: Raven.
- Gadbois DM, Crissmann HA, Tobey RA and Bradbury EM** (1992): Multiple kinase arrest points in the G1 phase of non-transformed mammalian cells are absent in transformed cells. *Proc. Natl. Acad. Sci. USA* 89: 8626-8630

- Garcia-Cardena G, Martasek P, Masters BS, Skidd PM, Couet J, Li S, Lisanti MP and Sessa WC (1997):** Dissecting the interaction between nitric oxide synthase (NOS) and caveolin. Functional significance of the NOS caveolin-binding domain in vivo. *J. Biol. Chem.* 272: 25437-25440
- Garcia-Ruiz C, Colell A, Mari M, Morales A and Fernandez-Checa JC (1997):** Direct effect of ceramide on the mitochondrial electron transport chain leads to generation of reactive oxygen species. Role of mitochondrial glutathione. *J Biol Chem.* 272: 11369-11377
- Garcia-Ruiz C, Mari M, Morales A, Colell A, Ardite E and Fernandez-Checa Jc (2000):** human placenta sphingomyelinase, an exogenous acidic pH-optimum sphingomyelinase, induces oxidative stress, glutathione depletion and apoptosis in rat hepatocytes. *Hepatol.* 32: 56-65
- Garthwaite J, Charles SL and Chess-Williams R (1988):** Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. *Nature* 336: 385-388
- Geng YJ, Hellstrand K, Wennmalm A and Hansson GK (1996):** Apoptotic death of human leukemic cells induced by vascular cells expressing nitric oxide synthase in response to gamma-interferon and tumor necrosis factor-alpha. *Cancer Res.* 56: 866-874
- Golstein P, Marguet D and Depraetere V (1995):** Homology between reaper and the cell death domains of Fas and TNFR1. *Cell* 81:185-186
- Gossen M and Bujard H (1992):** Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc. Natl. Acad. Sci. USA* 89:5547-5551
- Grassme H, Schwarz H and Gulbins (2001):** Molecular mechanism of ceramide mediated CD95 clustering. *Biochem. Biophys. Res. Commun.* 284: 1016-1030
- Green DR and Reed JC (1998)** Mitochondria and apoptosis. *Science* 281: 1309-1311
- Gross A, McDonnell JM and Korsmeyer SJ (1999):** BCL-2 family members and the mitochondria in apoptosis *Genes Dev.* 13: 1899-1911
- Gross SS, and Wolin MS (1995):** Nitric Oxide: Pathophysiological mechanism. *Annu.Rev.Physiol.* 57: 737-769
- Gruetter CA, Barry BK, McNamara DB, Gruetter DY, Kadowitz PJ, and Ignarro LJ (1979):** Relaxation of bovine coronary artery and activation of coronary arterial guanylate cyclase by nitric oxide, nitroprusside and a carcinogenic nitrosamine. *J. Cyclic Nucleotide Res.* 5: 211-224
- Gulbins E, Brenner B, Koppenhoefer U, Linderkamp O and Lang F (1998):** Fas or ceramide induce apoptosis by Ras-regulated phosphoinositide-3-kinase activation. *J. Leuc. Biol* 63: 253-263

- Hanna AN, Chan EY, Xu J, Stone JC and Brindley DN (1999):** A novel pathway for tumor necrosis factor- α and ceramide signaling involving sequential activation of tyrosine kinase, p21 (ras), and phosphatidylinositol 3-kinase. *J Biol Chem.* 274: 12722-12729
- Hannun YA (1996):** Functions of ceramide in coordinating cellular responses to stress. *Science* 274: 1855-1859
- Hannun YA, Luberto C and Argraves KM (2001):** Enzymes of sphingolipid metabolism: from modular to integrative signalling. *Biochemistry* 40: 4893-4903
- Harris MB, Ju H, Venema VJ, Liang H, Zou R, Michell RJ, Chen ZP, Kemp BE and Venema RC (2001):** Reciprocal phosphorylation and regulation of Endothelial Nitric-oxide synthase in response to bradykinin stimulation. *J. Biol. Chem.* 276: 16587-16591
- Hausmann G, O'Reilly LA, Van Driel R, Beaumont JG, Strasser A, Adams JM and Huang DCS (2000):** Pro-apoptotic Apoptosis Protease-activating Factor 1 (Apaf-1) has a cytoplasmic localization distinct from Bcl-2 or Bcl-xL. *J. Cell Biol.* 149: 623-634
- Hayashi Y, Nishio M, Naito Y, Yokokura H, Nimura Y, Hidaka H and Watanabe Y (1999):** Regulation of neuronal nitric-oxide synthase by calmodulin kinases. *J. Biol. Chem.* 274: 20597-20602
- Haynes MP, Sinha D, Russell KS, Collinge M, Fulton D, Morales-Ruiz M, Sessa WC and Bender JR (2000):** Membrane estrogen receptor engagement activates endothelial nitric oxide synthase via the Pi3K-Akt pathway in human endothelial cells. *Circ. Res* 87: 677-682
- Hecker M, Mulsch A, Bassengue E, Fostermann and Busse R (1994):** Subcellular localization and characterization of nitric oxide synthase(s) in endothelial cells: physiological implications. *Biochem. J.* 299: 247-252
- Hengarter MO and Horvitz HR (1994):** C.elegans cell survival gene ced-9 encodes a functional homologue of the mammalian proto-oncogene bcl-2. *Cell* 76: 665-676
- Hermann C, Zeiher AM and Dimmeler S (1997):** Shear stress inhibits H₂O₂ induced apoptosis of human endothelial cells by modulation of the glutathione redox cycle and nitric oxide synthase. *Arterioscl. Thromb. Vasc. Biol.* 17: 3588-3592
- Hibbs JBJr, Vavrin Z, Taintor RR (1987):** L-Arginine is required for expression of the activated macrophage effector mechanism causing selective metabolic inhibition in target cells. *J. Immunol.* 138: 550-565
- Higuchi M, Honda T, Proske RJ and Yeh ET (1998):** Regulation of reactive oxygen species-induced apoptosis and necrosis by caspase 3-like proteases. *Oncogene* 17: 2753-2760
- Higuchi M, Proske RJ and Yeh ET (1998):** Inhibition of mitochondrial respiratory chain complex I by TNF results in cytochrome c release, membrane permeability transition, and apoptosis. *Oncogene* 17: 2515-2524

- Higuchi M, Singh S, Jaffrezou JP and Aggarwal BB** (1996): Acidic sphingomyelinase-generated ceramide is needed but not sufficient for TNF-induced apoptosis and nuclear factor-kappa B activation. *J. Immunol.* 156: 297-304
- Hirata H, Takahashi A, Kobayashi S, Yonehara S, Sawai H, Okazaki T, Yamamoto K and Sasada** (1998): Caspases are activated in a branched protease cascade and control distinct downstream processes in FAS induced apoptosis. *J. Exp. Med.* 187: 587-600
- Hla T, Lee MJ, Ancellin N, Paik JH and Klux MJ** (2001): Lysophospholipids: receptor revelations. *Science* 294: 1875-1878
- Hofmann K, Tomiuk S, Wolff G, Stoffel W** (2000): Cloning and characterization of the mammalian brain-specific, Mg²⁺-dependent neutral sphingomyelinase. *Proc Natl Acad Sci U S A.* 97: 5895-5900
- Honig LS and Rosenberg RN** (2000): Apoptosis and neurological disease. *Am J Med* 108: 317-330
- Horinouchi K, Erlich S, Perl DP, Ferlinz K, Bisgaier CL, Sandhoff K, Desnick RJ, Stewart CL, Schuchman EH.** (1995): Acid sphingomyelinase deficient mice: a model of types A and B Niemann-Pick disease. *Nat Genet.* 10: 288-293
- Hortelano S, Dallaporta B, Zamzami N, Hirsch T, Susin SA, Marzo I, Bosca L and Kroemer G** (1997): Nitric oxide induces apoptosis via triggering mitochondrial permeability transition. *FEBS Lett.* 410: 373-377
- Horvitz HR** (1999): Genetic control of programmed cell death in the nematode *C. elegans*. *Cancer res.* 59: 1701s-1706s
- Hsu H, Shu HB, Pan MG and Goeddel DV** (1996a): TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. *Cell* 84:299-308
- Hsu H, Huang J, Shu HB, Baichwal V and Goeddel DV** (1996b): TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex. *Immunity.* 4:387-396
- Hsu H, Xiong J and Goeddel DV** (1995): The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. *Cell* 81: 495-504
- Huang B, Eberstadt M, Olejniczak ET, Meadows RP and Fesik SW** (1996): NMR structure and mutagenesis of the Fas (APO-1/CD95) death domain. *Nature* 384: 638-641
- Huang C, Ma WY, Ding M, Li J, Shi X, Castranova V, Vallyathan V, Bode AM and Dong Z** (2001): Involvement of sphingomyelinase in insulin-induced phosphatidylinositol 3-kinase activation. *FASEB J.* 15: 1113-1124

- Huwiler A, Kolter T, Pfeilshifter J and Sandhoff K** (2000): Physiology and pathophysiology of sphingolipid metabolism and signaling. *Biochim. Biophys. Acta* 1485: 63-69
- Ibitayo AI, Tsunoda Y, Nozu F, Owyang C and Bitar KN** (1998): Src kinase and PI 3-kinase as a transduction pathway in ceramide-induced contraction of colonic smooth muscle. *Am J Physiol.* 275: 705-711
- Igarashi J, Bernier SG and Michel T** (2001): Sphingosine-1-phosphate and activation of endothelial nitric-oxide synthase. Differential regulation of Akt and MAP kinase pathways by EDG and bradykinin receptors in vascular endothelial cells. *J. Biol. Chem.* 276: 12420-12426
- Igarashi J, Thatte HS, Prabhakar P, Golan DE and Michel T** (1999): Calcium-independent activation of endothelial nitric oxide synthase by ceramide. *Proc Natl Acad Sci U S A.* 96: 12583-12588
- Ignarro LJ** (1989): Endothelium derived nitric oxide: Pharmacology and relationship to the actions of organic nitrate esters. *Pharm.Res.* 6: 651-659
- Jensen JM, Schutze S, Forl M, Kronke M and Proksch E** (1999): Roles for tumor necrosis factor receptor p55 and sphingomyelinase in repairing the cutaneous permeability barrier. *J. Clin. Invest.* 104: 1761-1770
- Johns DG, Jin JS and Webb RC** (1998): The role of the endothelium in ceramide-induced vasodilation. *Eur J Pharmacol.* 349: R9-10
- Kawanaka H, Jones MK, Szabo IL, Baatar D, Pai R, Tsugawa K, Sugimachi K, Sarfeh IJ and Tarnawski AS** (2002): Activation of eNOS in rat portal hypertensive gastric mucosa is mediated by TNF-alpha via the PI 3-kinase-Akt signaling pathway. *Hepatology* 35: 393-402
- Kelekar A and Thomson CB** (1998): Bcl-2 homology domains: the role of the BH3 domain in apoptosis. *Trend Cell. Biol.* 8: 324-329
- Kerr JFR, Wyllie AH and Currie AR** (1972): Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics. *Br. J. Cancer* 26: 239-257
- Kim YM, Kim TH, Seol DW, Talanian RV and Billiar TR** (1998): Nitric oxide suppression of apoptosis occurs in association with an inhibition of Bcl-2 cleavage and cytochrome c release. *J Biol Chem.* 273: 31437-31441
- Kim YM, Talanian RV and Billiar TR** (1997): Nitric oxide inhibit apoptosis by preventing increases in caspase-3-like activity via two distinct mechanisms. *J. Biol. Chem.* 272: 31138-31148
- Kinscherf R, Wagner M, Kamencic H, Bonaterra GA, Hou D, Schiele RA, Deigner HP and Metz J** (1999): Characterization of apoptotic macrophages in atheromatous tissue of humans and heritable hyperlipidemic rabbits. *Atherosclerosis* 144: 33-39

- Klatt P, Schmidt K and Mayer B** (1992): Brain nitric oxide synthase is a haemoprotein. *Biochem. J.* 288:15-17
- Kolesnick RN** (1987): 1,2 Diacylglycerols but not phorbol esters, stimulate sphingomyelin hidrolysis in GH3 pituitary cells. *J. Biol. Chem.* 262: 16759-16762
- Kolesnick RN** (1989): Sphingomyelinase action inhibits phorbol ester induced differentiation of human promyelocytic leukemic (HL-60) cells. *J. Biol. Chem* 264: 7617-7623
- Kolesnick RN and Kronke M** (1998): Regulation of ceramide production and apoptosis. *Annu. Rev. Physiol.* 60: 643-665
- Kolodny EH** (2000) Niemann-Pick disease. *Curr. Opin. Hematol.* 7: 48-52
- Kone BC and Kuncewicz T** (1998): The Rho family GTPase Rac2 physically interacts with and activates inducible nitric oxide synthase. *J. Am. Soc. Nephrol.* 9: 463a
- Kothakota S, Azuma T, Reinhard C, Klippel A, Tang J, Chu K, McGarry TJ, Kirschner MW, Kothe K, Kwiatkowski DJ and Williams LT** (1997): Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. *Science.* 278: 294-298
- Lakics V and Vogel SN** (1998): Lipopolisaccharide and ceramide use divergent signalling pathways to induce cell death in murine macrophages. *J. Immunol.* 161: 2490-2500
- Levade T and Jaffrezou** (1999): Signalling sphingomyelinases: which, where, how and why? *Biophys. Acta* 1438: 1-17
- Li J, Bombeck CA, Yang S, Kim YN and Billiar TR** (1999): Nitric Oxide Suppresses Apoptosis via Interrupting Caspase Activation and Mitochondrial Dysfunction in Cultured Hepatocytes *J. Biol. Chem.* 274: 17325-17333
- Li J, Yang S and Billiar TR** (2000): Cyclic nucleotides suppress tumor necrosis factor α mediated apoptosis by inhibiting caspase activation and cytochrome c release in primary hepatocytes via a mechanism independent of Akt activation. *J. Biol. Chem.* 275: 13026-13034
- Li P, Nijhavan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES and Wang J** (1997): Cytochrome c and dATP-dependent formation of Apaf-1/caspase9 complex initiates an apoptotic protease cascade. *Cell* 91: 479-489
- Li S, Couet J and Lisanti MP** (1996): Src tyrosine kinases, G α subunits, and H-Ras share a common membrane anchored scaffolding protein, caveolin. *J. Biol. Chem.* 271: 29182-29910
- Liang H and Fesik SW** (1997): Three-dimensional structures of proteins involved in programmed cell death. *J. Mol. Biol.* 274: 291-302

- Lievremont JP, Sciorati C, Morandi E, Paolucci C, Bunone G, Della Valle G, Meldolesi J and Clementi E** (1999): The p75 (NTR)-induced apoptotic program develops through a ceramide caspase pathway negatively regulated by nitric oxide. *J. Biol. Chem.* 274: 15466-15472
- Lin X, Fuks Z, Kolesnick R.** (2000): Ceramide mediates radiation-induced death of endothelium. *Crit Care Med.* 28(4 Suppl): N87-93
- Liu B, Andrieu-Abadie N, Levade T, Zhang P, Obeid LM and Hannun YA** (1998): Glutathione regulation of neutral sphingomyelinase in tumor necrosis factor- α induced cell death. *J. Biol. Chem.* 273: 11313-11320
- Liu L and Stamler JS** (1999): NO: an inhibitor of cell death. *Cell Death Diff.* 6: 937-942
- Liu P and Anderson RGW** (1995): Compartmentalized production of ceramide at the cell surface. *J. Biol. Chem.* 270: 27179-27185
- Liu X, Kim CN, Yang J, Jammerson R and Wang X** (1996): Induction of apoptotic program in cell free extracts: requirement for dATP and cytochrome c. *Cell* 86: 147-156
- Liu ZG, Hsu H, Goeddel D, and Karin M** (1996): Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF- κ B activation prevent cell death. *Cell* 87:565-576
- Lozano J, Menendez S, Morales A, Ehleiter D, Liao WC, Wagman R, Haimovitz-Friedman A, Fuks Z and Kolesnick R** (2001): Cell autonomous apoptosis defect in acid sphingomyelinase knockout fibroblasts. *J. Biol. Chem.* 276: 442-448
- Lozano J, Menendez S, Morales, Ehleiter D, Liao WC, Wagman R, Haimovitz-Friedman A, Fuks Z and Kolesnick R** (2001): Cell autonomous apoptosis defects in acid sphingomyelinases knockout fibroblasts. *J. Biol. Chem.* 276: 442-448
- Luberto C, Hassler DF, Signorelli P, Okamoto Y, Sawai H, Boros E, Hazen-Martin DJ, Obeid LM, Hannun YA and Smith GK** (2002): Inhibition of TNF-induced cell death in MCF7 by a novel inhibitor of neutral sphingomyelinase. *J Biol Chem.* [epub ahead of print; still in press at the moment]
- MacMicking J, Xie QW and Nathan C** (1997): Nitric oxide and macrophage function. *Annu Rev Immunol.* 15: 323-350
- Mannick JB, Asano K, Izumi K, Kieff E and Stamler JS** (1994): Nitric oxide produced by human B lymphocytes inhibit apoptosis and Epstein-Barr virus reactivation. *Cell* 79: 1137-1146
- Mannick JB, Miao XQ and Stamler JS** (1997): Nitric Oxide inhibits Fas-induced apoptosis. *J. Biol. Chem.* 272: 24125-24128
- Mansat V, Laurent G, Levade T, Bettaieb A and Jaffrezou JP** (1997): The protein kinase C activators phorbol esters and phosphatidylserine inhibit neutral sphingomyelinase

- activation, ceramide generation and apoptosis triggered by daunorubicin. *Cancer Res* 57: 5300-5304
- Martin SJ, Newmeyer DD, Mathias S, Farschon DM, Wang HG, Reed JC, Kolesnick RN and Green DR** (1995): Cell-free reconstitution of Fas-, UV radiation- and ceramide-induced apoptosis. *EMBO J* 14:5191-5200
- Mathias S, Pena LA and Kolesnick RN** (1998): Signal transduction via ceramide. *Biochem. J.* 335:465-480
- McCabe TJ, Fulton D, Roman LJ and Sessa WC** (2000): enhanced electron flux and reduced calmodulin dissociation may explain «calcium independent» eNOS by phosphorylation. *J. Biol. Chem.* 275: 6123-6128
- Messmer UK, Reed JC and Brune B** (1996): Bcl-2 protects macrophages from nitric oxide induced apoptosis. *J. Biol. Chem.* 271: 20192-20197
- Michel JB, Feron O, Sacks D and Michel T** (1997): Reciprocal regulation of endothelial nitric-oxide synthase by Ca²⁺-calmodulin and caveolin. *J. Biol. Chem.* 272: 15583-15586
- Michel JB, Feron O, Sase P, Prabhakar P and Michel T** (1997): Caveolin versus Calmodulin: counterbalancing allosteric modulators of nitric oxide synthase. *J. Biol. Chem.* 272: 25907-25912
- Moncada S and Higgs EA** (1995): Molecular mechanisms and therapeutic strategies related to nitric oxide. *FASEB J.* 9: 1319-1330
- Moncada S, Palmer RM, and Higgs EA** (1991): Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol.Rev.* 43: 109-142
- Monick MM, Mallampalli RK, Carter AB, Flaherty DM, McCoy D, Robeff PK, Peterson MW and Hunninghake GW** (2001): Ceramide regulates lipopolysaccharide-induced phosphatidylinositol 3-kinase and Akt activity in human alveolar macrophages. *J Immunol.* 167: 5977-5985
- Montagnani M, Chen, Barr VA and Quon MJ** (2001): Insulin-stimulated activation of eNOS is independent of Ca²⁺ but requires phosphorylation by Akt at Ser (1179). *J. Biol. Chem.* 276: 30392-30398
- Moriishi K, Huang DCS, Cory S, and Adams JM** (1999): Bcl-2 family members do not inhibit apoptosis by binding the caspase activator Apaf-1. *Proc. Nat. Acad. Sci. USA* 96: 9683-9688
- Muzio M, Stockwell BR, Stennicke HR, Salvesen GS and Dixit VM** (1998): An induced proximity model for caspase-8 activation. *J Biol Chem.* 273: 2926-2930
- Nagata S** (1997): Apoptosis by death factor. *Cell* 88: 355-365
- Nathan C and Xie QW** (1994): Nitric oxide synthases: roles, tolls and controls. *Cell* 78: 915-918

- Newton K, Harris AW, Bath ML, Smith KGC and Strasser A** (1998): A dominant interfering mutant of FADD/MORT1 enhances deletion of autoreactive thymocytes and inhibits proliferation of mature T lymphocytes. *EMBO J.* 17: 706-718
- Nicholson DW and Thornberry NA** (1997): Caspases: killer proteases. *Trends Biochem Sci.* 8: 299-306
- Nishikawa M, Sato EF, Kuroki T, Utsumi K and Inoue M** (1998): Macrophage derived nitric oxide induces apoptosis of rat hepatoma cells in vivo. *Hepatology* 28: 1474-1480
- Nishizuka Y** (1995): Protein kinase C and lipid signaling for sustained cellular responses. *FASEB J.* 9:484-496
- Ogura T, DeGeorge T, Tatemichi M and Esumii H** (1998): Suppression of anti-microtubule agent induced apoptosis by nitric oxide: possible mechanism of a new drug resistance. *Jpn. J. Cancer Res.* 89: 199-205
- Okazaki T, Bielawska A, Bell RM and Hannun YA** (1990): Role of ceramide as a lipid mediator of 1°,25-dihydroxyvitamin D3-induced HL-60 cell differentiation. *J. Biol. Chem.* 265: 15823-15831
- Olivera A and Spiegel S** (1993): Sphingosine-1-phosphate as 2nd messenger in cell proliferation. *Nature* 365: 557-560
- Osborne B.A** (1996): Apoptosis and the maintenance of homoeostasis in the immune system. *Curr. Opin. Immunol.* 2: 245-254
- Ou J, Carlos TM, Watkins SC, Saavedra JE, Keefer LK, Kim YM, Harbrecht BG and Billiar** (1997): Differential effects of non-selective nitric oxide synthase(NOS) and selective inducible NOS inhibition on hepatic necrosis, apoptosis, ICAM-1 expression and neutrophil accumulation during endotoxemia. *Nitric Oxide* 1: 404-416
- Palmer RM, Ashton DS and Moncada S** (1988): Vascular endothelial cells synthesize nitric oxide from L-Arginine. *Nature* 333: 664-666
- Palmer RMJ, Ferridge AG and Moncada S** (1987): Nitric Oxide release accounts for the biological activity of endothelium derived relaxing factor. *Nature* 327: 524-526
- Pastorino JG, Simbula G, Yamamoto K, Glascott PA Jr, Rothman RJ and Farber JL** (1996): The cytotoxicity of tumor necrosis factor depends on induction of the mitochondrial permeability transition. *J Biol Chem.* 271: 29792-29798
- Perry DK** (2000): The role of de novo ceramide synthesis in chemotherapy-induced apoptosis. *Ann N Y Acad Sci.* 905: 91-96
- Perry DK and Hannun Y** (1998): The role of ceramide in cell signalling. *Biochem. Biophys. Acta* 1436: 233-243
- Piomelli D** (1993): Arachidonic acid in cell signaling. *Curr Opin Cell Biol.* 5: 274-80

- Plo I, Bettaieb A, Payraastre B, Mansat-De Mas V, Bordier C, Rousse A, Kowalski-Chauvel A, Laurent G and Lautier D** (1999): The phosphoinositide 3-kinase/Akt pathway is activated by daunorubicin in human acute myeloid leukemia cell lines. *FEBS Lett.* 452: 150-154
- Raff M** (1998) Cell suicide for beginners *Nature* 396: 119-122
- Richter C, Schweizer M, Cossarizza A and Franceschi C** (1996): Control of apoptosis by the cellular ATP level. *FEBS Lett.* 378: 107-110
- Rodriguez-Lafrasse C and Vaniere MT** (1999): Sphingosyl phosphorylcholine in Niemann-Pick disease brain: accumulation in type A but not type B. *Neurochem. Res.* 24: 199-205
- Rothe M, Wong SC, Henzel WJ and Goeddel DV** (1994): A novel family of putative signal transducers associated with the cytoplasmic domain of the 75-kDa-tumour necrosis factor receptor. *Cell* 78: 681-692
- Saavedra JE, Billiar TR, Williams DL, Kim YM, Watkins SC and Keefer LK** (1997): Targeting nitric oxide (NO) in vivo. Design of a liver-selective NO donor prodrug that blocks tumor necrosis factor alpha induced apoptosis and toxicity in the liver. *J. Med. Chem.* 40: 1947-1954
- Salvesen GS and Dixit VM** (1999): Caspase activation: the induced proximity model. *Proc. Natl. Acad. Sci USA* 96: 10964-10967
- Santana P, Pena LA, Haimovitz-Friedman A, Martin S, Green D, McLoughlin M, Cordon-Cardo C, Schuchman EH, Fuks Z and Kolesnick R** (1996): Acid sphingomyelinase-deficient human lymphoblasts and mice are defective in radiation-induced apoptosis. *Cell.* 86:189-199
- Sarih M, Souvannavong V and Adam A** (1993): Nitric oxide induces macrophage death by apoptosis. *Biochem. Biophys. Res. Commun.* 191: 503-508
- Sattler M, Liang H, Nettesheim D, Meadows RP, Harlan JE, Eberstadt M, Yoon HS, Shuker SB, Chang BS, Minn AJ, Thompson CB and Fesik SJ** (1997): Structure of Bcl-xl-Bak peptide complex: recognition between regulators of apoptosis. *Science* 275: 983-986
- Schissel SL, Jiang X, Tweedie-Hardman J, Jeong TS, Camejo EH, Najib J, Rapp JH, Williams KJ and Tabas I** (1998): Secretory sphingomyelinase, a product of the acidic sphingomyelinases gene can hydrolyze atherogenic lipoproteins at neutral pH. Implications for atherosclerotic lesion development. *J. Biol. Chem.* 273: 2738-2746
- Schutze S, Machleid T, Adam D, Schwandner R, Wiegmann K, Kruse ML, Heinrich M, Wickel M and Kronke M** (1999): Inhibition of receptor internalization by monodansylcadaverine selectively blocks p55 tumor necrosis factor receptor death domain signalling. *J. Biol. Chem.* 274: 10203-10212

- Schwander SK, Torres M, Sada E, Carranza C, Ramos E, Tary-Lehmann M, Wallis RS, Sierra J and Rich EA** (1998): Enhanced responses to Mycobacterium tuberculosis antigens by human alveolar lymphocytes during active pulmonary tuberculosis. *J Infect Dis.* 178:1434-1445
- Sciorati C, Rovere P, Ferrarini M, Heltai S, Manfredi AA and Clementi E** (1997): Autocrine nitric oxide modulates CD95-induced apoptosis in gamma delta T lymphocytes. *J. Biol. Chem.* 272: 23211-23215
- Segui B, Cuvillier O, Adam-Klages S, Garcia V, Malagarie-Cazenave S, Leveque S, Caspar-Bauguil S, Coudert J, Salvayre R, Kronke M and Levade T** (2001): Involvement of FAN in TNF-induced apoptosis. *J Clin Invest* 108: 143-151
- Sessa WC** (1994): The Nitric Oxide Synthase family of proteins. *J.Vasc. Res.* 31: 131-143
- Sessa WC, Garcia-Cardena G, Liu J, Keh A, Pollock JS, Bradley J, Thiru S, Braverman IM and Deasi KM** (1995): The Golgi association of endothelial nitric oxide synthase is necessary for efficient synthesis of nitric oxide. *J. Biol. Chem.* 270:17641-17644
- Sessa WC, García-cardena G, Liu J, Keh A, Pollock JS, Bradley J, Thiru S, Braverman IM and Deasi KM** (1995): The Golgi association of endothelial nitric oxide synthase is necessary for the efficient synthesis of nitric oxide *J. Biol. Chem.* 270: 17641-17644
- Sessa WC, Harrison JK, Barber CM, Zeng D, Durieux ME, D'Angelo DD, Lynch KR and Peach MJ.** (1992): Molecular cloning and expression of a cDNA encoding endothelial cell nitric oxide synthase. *J Biol Chem.* 267:15274-15276
- Sestili P, Cattabeni F and Cantoni O** (1996): Direct excision of 50 kb pair DNA fragments from megabase-sized fragments produced during apoptotic cleavage of genomic DNA, *FEBS Letters* 396: 337-342
- Shaul PW** (2002): Regulation of endothelial nitric oxide synthase: Location, location, location. *Ann. Rev. Physiol.* 64: 749-774
- Shaul PW, Smart EJ, Robinson LJ, German Z, Yuhanna IS, Ying Y, Anderson RG and Michel T** (1996): Acylation targets endothelial nitric-oxide synthase to plasmalemmal caveolae *J Biol. Chem.* 271:6518-6522
- Shirakabe K, Yamaguchi K, Shibuya H, Irie K, Matsuda S, Moriguchi T, Gotoh Y, Matsumoto K and Nishida E** (1997): TAK1 Mediates the Ceramide Signaling to Stress-activated Protein Kinase/c-Jun N-terminal Kinase *J. Biol. Chem.* 272: 8141-8144
- Sowa G, Pypaert M and Sessa WC** (2001): Distinction between signaling mechanisms in lipid rafts vs. caveolae. *Proc Natl Acad Sci U S A.* 98:14072-14077
- Spiegel S, Foster D, Kolesnick R** (1996): Signal transduction through lipid second messengers. *Curr Opin Cell Biol.* 8:159-167

- Srinivasula SM, Ahmad , Fernades-Alnemri T and Alnemri ES** (1998): Autoactivation of procaspase-9 by Apaf-1 mediated oligomerization. *Mol. Cell.* 1: 949-957
- Stasch JP, Becker EM, Alonso-Alija C, Apeler H, Dembosky K, Feuer A, Gerzer R, Minuth T, Perzborn E, Pleiss U, Schroder H, Schroder W, Stahl E, Steinke W, Straub A and Schramm M** (2001): NO independent regulatory site on soluble guanylate cyclase. *Nature* 410: 212-215
- Stuehr DJ** (1999): Mammalian nitric oxide synthases. *Biochim. Biophys. Acta* 1411: 217-230
- Stuehr DJ and Nathan CF** (1989): Nitric oxide: a macrophage product responsible for the cytostasis and respiratory inhibition in tumor target cells. *J.Exp. Med.* 169: 1543-1555
- Takuma K, Phuagphong P, Lee E, Mori K, Baba A and Matsuda T** (2001): Anti-apoptotic effect of cGMP in cultured astrocytes: inhibition by cGMP-dependent protein kinase of mitochondrial permeable transition pore. *J. Biol. Chem.* 276: 48093-48099
- Tanaka M, Nara F, Suzuki-konagai K, Hosoya T and Ogita T** (1997): Structural elucidation of scyphostatin, an inhibitor of membrane-bound neutral sphingomyelinase. *J. Am. Chem. Soc.* 116: 7871-7872
- Tartaglia LA, Ayres TM, Wong GH and Goeddel W** (1993): A Novel Domain within the 55 Kd TNF Receptor Signals Cell Death. *Cell* 74: 845-853
- Thornberry NA and Lazebnik Y** (1998): Caspases: enemies within. *Science.* 281: 1312-1316
- Thornberry NA, Rano TA, Peterson EP, Rasper DM, Timkey T, Garcia-Calvo M, Houtzager VM, Nordstrom PA, Roy S, Vaillancourt JP, Chapman KT and Nicholson DW** (1997): A combinatorial approach defines specificities of members of the caspase family and granzyme-B. *J. Biol. Chem.* 272: 17907-17922
- Van Antwerp DJ, Martin SJ, Kafri T, Green DR and Verma IM** (1996): Suppression of TNF-alpha-induced apoptosis by NF-kappaB. *Science.* 274:787-789
- Vander Heiden MG, Chandel NS, Li XX, Schumacher PT, Colombini M and Thompson CB** (2000): Outer mitochondrial membrane permeability can regulate coupled respiration and cell survival. *Proc Nat Acad Sci USA* 97: 4666-4671
- Vander Heiden MG, Chandel NS, Williamson EK, Schumacker PT and Thompson CB** (1997): Bcl-xL Regulates the Membrane Potential and Volume Homeostasis of Mitochondria *Cell* 91: 627-637
- Vaux DL, Cory S and Adams JM** (1988): Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B-cells. *Nature* 335: 440-442
- Vaux DL, Weissman IL and Kim SK** (1992) Prevention of programmed cell death in *Caenorhabditis elegans* by human bcl-2. *Science* 258:1955-1957

- Veldman RJ, Maestre N, Aduib OM, Medin JA, Salvayre R and Levade T (2001):** A neutral sphingomyelinase resides in sphingolipid-enriched microdomains and is inhibited by the caveolin-scaffolding domain: potential implications in tumour necrosis factor signalling. *Biochem J.* 355: 859-868
- Verheij M, Bose R, Lin XH, Yao B, Jarvis WD, Grant S, Birrer MJ, Szabo E, Zon LI, Kyriakis JM, Haimovitz-Friedman A, Fuks Z and Kolesnick RN (1996):** Requirement for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis. *Nature* 380: 75-79
- Von Ahsen O, Renken C, Perkins G, Kluck RM, Bossy-Wetzel E and Newmeyer DD (2000):** Preservation of mitochondrial structure and function after Bid- or Bax-mediated Cytochrome c release *J. Cell Biol.* 150: 1027-1036
- Wang JS, Tseng HH, Shih DF, Jou HS and Ger LP (1997):** Expression of inducible nitric oxide synthase and apoptosis in human lupus nephritis. *Nephron* 77: 404-411
- Wang Y, Falasca M, Schlessinger J, Malstrom S, Tsichlis P, Settleman J, Hu W, Lim B and Prywes R (1998):** Activation of the c-fos serum response element by phosphatidylinositol 3-kinase and rho pathways in HeLa cells. *Cell Growth Differ.* 9:513-522
- Wen LP, Fahrni JA, Troie S, Guan JL, Orth K and Rosen GD (1997):** Cleavage of Focal Adhesion Kinase by caspases during apoptosis. *J. Biol. Chem.* 272: 26056-26061
- Wiegmann K, Schutze S, Machleid T, Witte D and Kronke M (1994):** Functional dichotomy of neutral and acidic sphingomyelinases in tumor necrosis factor signaling. *Cell* 78: 1005-1015
- Williams MS, Noguchi S, Henkart PA and Osawa Y (1998):** Nitric oxide synthase plays a significant role in TCR triggered apoptotic cell death. *J. Immunol.* 161: 6526-6531
- Williams TE, Ayala A and Chaudry IH (1997):** Inducible macrophage apoptosis following sepsis is mediated by cysteine protease activation and nitric oxide release. *J. Surg. Res.* 70: 113-118
- Wink KA, and Mitchell JB (1998):** Chemical biology of nitric oxide: Insight into regulatory, cytotoxic, and cytoprotective mechanism of nitric oxide. *Free Radicals Biol.Med.* 25: 434-456
- Xie K, Huang S, Dong Z and Fidler IJ (1993):** Cytokine induced apoptosis in transformed murine fibroblasts involves synthesis of endogenous nitric oxide. *Int. J. Oncol.* 3:1043-1048
- Xie KP, Huang SY, Dong ZY, Juang SH, Wang YF and Fidler IJ (1997):** Destruction of bystander cells by tumour cells transfected with inducible nitric oxide (NO) synthase gene, *J. Natl. Cancer Inst.* 89: 421-427
- Xie QW, Cho HJ, Calaycay J, Mumford RA, Swiderek KM, Lee TD, Ding A, Troso T and Nathan C (1992):** Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. *Science* 256: 225-228

- Yasuda M, Theodorakis P, Subramanian T and Chinnadurai G** (1998): Adenovirus E1B-19K/BCL-2 interacting protein BNIP3 contains a BH3 domain and a mitochondrial targeting sequence. *J Biol Chem.* 273:12415-12421
- Yeh WC, Shahinian A, Speiser D, Kraunus J, Billia F, Wakeham A, De La Pompa JL, Ferrick D, Hum B, Iscove N, Ohashi P, Rothe M, Goeddel DV and Mak TW** (1997): Early lethality, functional NF-kB activation, and increased sensitivity to TNF-induced cell death in TRAF2-deficient mice. *Immunity* 7: 715-725
- Yuan JS, Shaham S, Ledoux S, Ellis HM and Horvitz HR** (1993): The C.Elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1-beta- converting enzyme. *Cell* 75: 641-652
- Zamora R, Alarcon L, Vodovotz Y, Betten B, Kim PKM, Gibson KF and Billiar TR** (2001): Nitric Oxide suppresses the expression of Bcl-2 binding protein BNIP3 in hepatocytes. *J. Biol. Chem* 276: 46887-46895
- Zamora R, Vodovotz V, Alarcon L, Betten B, Loughran PA, Aulak KS, Stuehr DJ, Gibson KF and Billiar TR** (2001): Nitric Oxide from the Inducible Nitric Oxide Synthase (iNOS) Increases the Expression of Cytochrome P450 2E1 in iNOS-Null Hepatocytes in the Absence of Inflammatory Stimuli *Arch Biochem Biophys.* 390: 287-294
- Zhang J, Cado D, Chen A, Kabra NH and Winoto A** (1998): Fas-mediated apoptosis and activation-induced T-cell proliferation are defective in mice lacking FADD/Mort1. *Nature* 392:296-300
- Zheng TS and Flavell RA** (2000): Divinations and surprises: genetic analysis of caspase function in mice. *Exp. Cell Res.* 256: 67-73
- Zou H, Henzel WJ, Liu X, Lutschg A and Wang X** (1997) Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3 *Cell* 90: 405-413

ACKNOWLEDGEMENTS

I want to thank Jacopo Meldolesi, for the scientific supervision and mostly for being such a great scientist; thanks to Emilio Clementi for the precious guidance and direct supervision during these years, to Guy Brown for the time spent for critical discussion during his visits and for the patience to answer to all my e-mails and to Nica Borgese for being such an acute (and intense ☺) collaborator.

Thanks to all the people in the lab that helped me in these years in various ways, even only with being there: Cristiana for the help during this last year, the three Claras (P, S and DP), Sestina, Emanuele, Annina, Paola, Francesca, Raffaele, Andrea and all the others (you're just too many to name ☺).

Special thanks to Gabriella for being the “iron sergeant” and the “great mother” of the lab. Finally, to my family, Barbara and Napo: I love you.

APPENDIX: PUBLICATIONS

Papers:

DeNadai C, Sestili P, Cantoni O, Lievremon JP, Sciorati C, **Barsacchi R**, Moncada S, Meldolesi J and Clementi E (2000): Nitric oxide inhibits tumor necrosis factor α induced apoptosis by reducing the generation of ceramide. *Proc. Natl. Acad. Sci. USA* 97: 5480-5485

Bulotta S, **Barsacchi R** (co-authors), Rotiroti D, Borgese N and Clementi E (2001): Activation of endothelial nitric oxide synthase by tumor necrosis factor- α . *J. Biol. Chem.* 276: 6529-6536.

Barsacchi R, Perrotta C, Sestili P, Cantoni O, Moncada S and Clementi E (2002) cyclicGMP-dependent inhibition of acid sphingomyelinase by nitric oxide: an early step in protection against apoptosis. *Cell Death Diff.* 9: 1248-1255.

Book Chapters:

Barsacchi R, Sciorati C and Clementi E (2002) The cross talk between nitric oxide and ceramide and its role in apoptosis regulation. Chapter 13 of the book "Ceramide Signalling", edited by Anthony Futherman and published by Landes Bioscience, Georgetown, TX (USA)